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INTERINDIVIDUAL VARIABILITY IN THE CYTOCHROME P450 3A4 DRUG METABOLIZING ENZYME: EFFECT OF THE *CYP3A4*1G* GENETIC VARIANT

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Researchers and clinicians are interested in how a patient's individual genetic makeup could predict the appropriate medication and dose for that patient. One way to predict drug response, or efficacy, is by looking at enzymes within the liver that metabolize drugs. Many of these enzymes belong to a class called the Cytochrome P450s (CYPs). Specifically, two closely related enzymes, CYP3A4 and CYP3A5, are involved in metabolizing 50% of drugs currently on the market (eg: statins, antiepileptics, anticancer agents, and antidepressants). There can be differences in the genetic code of these enzymes that can causes changes in drug metabolism.

We completed a study with participants from the Confederated Salish and Kootenai Tribes (CSKT), located on the Flathead Reservation in northwest Montana. Select CYP enzymes were genotyped, including *CYP3A4* and *CYP3A5*. Most SNPs identified in the CSKT participants were found at frequencies similar to those reported in European-descended populations. Interestingly, one specific SNP, called *CYP3A4*1G*, was discovered at a high allele frequency. The physiological significance of this SNP is unclear as there are limited and confounding data, however, most of the data published to date suggest that the SNP causes decreased metabolism of drugs. Clinically, this could result in a need for a decreased dose of medication. In addition, this *CYP3A4* SNP was observed to be often inherited with another SNP in the related *CYP3A5* gene, called *CYP3A5*3*, which encodes a nonfunctional enzyme. These SNPs found in the CSKT are of particular interest, because inheriting these two SNPs together could cause drastic changes in drug metabolism since the two enzymes metabolize many of the same drugs.

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Chapter 1: Introduction

1.A. The Promise of Pharmacogenomics

Pharmacogenomics offers a new way of practicing medicine by individualizing medications and dosages based on an individual's genetic make-up [1, 2]. The goal is to optimize efficacy while minimizing adverse events [3]. The completion of the Human Genome Project in 2000 allowed scientists to more easily link specific genetic changes to differences in drug response and toxicity [1, 2].

The Federal Drug Administration (FDA) states that its "mission is to protect and promote the health of all Americans through assuring the safety, efficacy, and security of drugs…" [4]. The FDA believes personalized medicine has potential to increase efficacy and decrease risk of adverse drug reactions [4]. They have released guidelines to better integrate genetic information with medications [4-6] [3]. These are guidelines for new drug applications as well as when, how, and what pharmacogenomic data to submit [3]. They have also required that pharmacogenomic data be included in the product insert of 140 different medications, many with more than one predictive biomarker; the importance of these biomarkers can vary from drug choice, to dosage, to black box warnings [4]. Medications with pharmacogenomic data in the product insert are widespread: trastuzumab (oncology), tamoxifen (oncology), phenytoin (neurology), warfarin (hematology/cardiology), clopidogrel (cardiology), abacavir (infectious diseases), atorvastatin (endocrinology), codeine (anesthesiology), and fluoxetine (psychiatry). This information is available to the public on their website under the Table of Pharmacogenomic Biomarkers in Drug Labeling [7]. Also in 2007, the

FDA approved the first genotyping test, a DNA microarray, Amplichip CYP450. This is used by physicians to assist in medication and dosage choices [8].

Another organization that releases pharmacogenetics-guided dosing recommendations is the Clinical Pharmacogenetics Implementation Consortium (CPIC). The CPIC was formed in 2009 and focuses on providing guidelines designed to help providers understand how genetic test results should be used to optimize drug therapy in clinical practice [9]. To date, they have identified 174 gene-drug pairs of interest, which include 63 unique genes and 131 unique drugs. CPIC has published guidelines on 33 of these pairs. These 174 gene-drug pairs are broken down into levels (A, B, C, or D) that indicate level of evidence and strength of recommendation. Level A indicates there is evidence to change prescribing regimen of drug, while Level D indicates there is weak or conflicting evidence and no changes in the prescribing regimen are recommended at this time. Codeine, phenytoin, simvastatin, and warfarin are considered Level A. Fluoxetine, tamoxifen, and omeprazole are considered Level B. Level C drugs include propranolol and diazepam, and Level D drugs include aspirin, atorvastatin, caffeine, and metformin [10].

One CPIC Level A drug is tacrolimus, a medication given to patients who undergo solid organ or hematopoietic cell transplantation to prevent rejection. Tacrolimus has a very narrow therapeutic window and plasma levels are strictly monitored by therapeutic drug monitoring; too little drug leads to organ or graft

rejection while too much drug leads to nephrotoxicity [11-13]. In spite of individual monitoring, patients still experience lack of efficacy or adverse events. The clearance of tacrolimus is mediated by drug-metabolizing enzymes, cytochrome P450 3A4 (CYP3A4) and 3A5 (CYP3A5), which determine drug levels in the body. *In vitro* and *in vivo* data show that individuals with genotypes encoding for deleterious CYP3A5 enzyme function have lower clearances and higher trough concentrations than those expressing wildtype enzyme [14-18]. Using *CYP3A4* and *CYP3A5* genotypes to more accurately determine tacrolimus dosage regimens can improve efficacy through less dosage modifications and quicker time to target tacrolimus plasma concentrations [19].

1.A.i. Pharmacogenetics: Improving Outcomes

Most medications used today are efficacious in only 25% (oncology medications) to 80% (analgesic medications) of patients [4, 20]. Although efficacy can be affected by several factors such as patient compliance, diet, and drug interactions, genetic variations can play a large role [3]. Identifying patients at risk for adverse events can help to minimize injuries as well as reduce medical costs. Adverse drug reactions (ADRs) refer to significant side effect(s) of medications, some of which can be life threatening [21-23]. The Institute of Medicine reports that there are at least 1.5 million preventable ADRs in the United States (US) per year and they are considered the leading cause of preventable death [24, 25]. As a result of ADRs, there are more than 100,000 deaths per year in the US costing $100 billion per year [26]. Pharmacogenomics may be able to play a role in

reducing these adverse events by predicting those at higher risk due to a change in drug metabolism.

Interindividual variability in drug response and toxicity is multifactorial and include both extrinsic and intrinsic factors. Extrinsic factors include the environment (i.e. smoking, diet, and alcohol consumption) and drug interactions (i.e. concomitant use of other prescription medications, over-the-counter medications, and herbal supplements). Intrinsic factors include demographics (age, gender, and ethnicity) and disease (particularly liver and kidney dysfunction). Finally, genetic variation between individuals can also be a key player in differences in response and toxicity [27-30]. It has been proposed that genetic factors can account for as much as 20-95% of interindividual variability in drug disposition [31]. This wide estimation accounts for different effects of genetic factors in different gene-drug pairs. The amount of interindividual variability can be drug specific; it will affect metabolism rates differently depending on the drug given. Also, drug elimination pathways can be very complex, causing genetic factors to have different effects.

1.A.ii. Genotype-Phenotype Associations

Genotype-phenotype association studies are important in order to make a prediction about how a patient's genetic variation, or their genotype, can affect the response or toxicity to a given medication, known as their phenotype. Genetic variation can affect the outcome of about a quarter of all medications [29]. It is

important to identify individuals carrying these variations so that alternative medications or doses may be chosen.

Most genotype-phenotype correlations can be measured through *in vivo* or *in vitro* probe drug assays [32]. Probe drugs are metabolized by a single drug-metabolizing enzyme and are administered to identify the function of that enzyme. Phenotypes can be measured through administration of a subtherapeutic dose of probe drug [33]. When using probe drugs to measure a phenotype, plasma and urine concentrations of the parent drug and metabolite are measured in order to estimate the pharmacokinetics of the parent and metabolite(s) [33, 34]. An advantage of this method is that phenotype is directly measured under current conditions (diet, age, disease state, etc). However, there are several disadvantages. This method can have complicated protocols and there is a risk of determining the wrong phenotype to do concurrent medications or disease state. There is also a risk of an ADR in patients of extreme phenotypes [34]. However, in clinical practice, phenotype is typically measured by a clinical outcome (i.e. INR for warfarin or reducing LDL levels for statins) [35].

There are four phenotypes: Poor Metabolizers (PM), Intermediate Metabolizers (IM), Extensive Metabolizers (EM), and Ultra Metabolizers (UM). PMs do not express active enzyme. This can cause increase risk of toxicity if the medication is toxic but decreased efficacy if metabolite is active. IMs have reduced enzyme activity. These patients continue to have lower metabolism than the standard

population. EMs express fully active enzyme and standard doses are given to these patients. UMs have multiple copies of functional enzyme. These patients may have increased risk for toxicity if the metabolite is toxic or decreased efficacy if the parent medication is active [8, 33, 34, 36].

Genotyping allows practitioners to optimize the drug choice and dose for each individual and avoid most ADRs. These can lead to decreased medical costs [34]. Genotyping can have the largest clinical impact on patients taking narrow therapeutic medications, patients with unexplained side effects, as well as, older patients. Older patients tend to take more medications and are more likely to exhibit serious side effects; they also can display large changes in metabolism due to decreased liver and kidney function [37].

1.A.iii. Sources of Genetic Variability in Drug Response and Toxicity

Pharmacogenetic variability results from genetic variation in both pharmacokinetic and pharmacodynamic properties of a drug. Pharmacokinetics is the study of the effect of the body on drugs, while pharmacodynamics is the study of the effects of drugs on the body. Alterations in pharmacokinetic pathways alter the drug exposure in an individual and make up the vast majority of pharmacogenetic variability.

The pharmacokinetic disposition of a xenobiotic can be broken down by the processes of absorption, distribution, metabolism, and elimination (ADME) [38].

Absorption describes the processes that control the rate and extent of absorption of a compound from the site of administration to the systemic circulation. These processes include passive diffusion (small, lipophilic xenobiotics), active or facilitated transport (large, polar, or charged xenobiotics), and first-pass extraction in the gastrointestinal tract and liver for drugs that are orally administered [39]. After xenobiotics have entered systemic circulation, they are distributed from the vasculature to various tissues of the body, including those where they exert pharmacologic, and perhaps toxic, effect. The extent of this distribution depends upon passive and active diffusion rates across membranes and protein binding in both blood and tissues [38]. Metabolism mainly occurs in the liver, however, other tissues are known to have some metabolism such as kidney, lungs, and intestines [40]. Substrates undergo a wide range of metabolic reactions. Most undergo Phase I metabolism where the substrate undergoes oxidation, reduction, or hydrolysis reactions. Next, drugs often undergo sequential Phase II metabolism, which are conjugative reactions [38]. Hepatic metabolism is a primary component in the clearance of many compounds. Metabolism also plays a large role in first-pass extraction in the intestine and liver and, consequently, has a large effect on bioavailability of compounds that are extensively metabolized [38, 41]. Elimination of xenobiotics includes both the processes of metabolism and excretion. The primary routes of excretion are biliary excretion, via transporters, in the liver [42] and urinary excretion in the kidneys, by glomerular filtration and passive and active secretion via transporters [38, 43].

Phase I enzymes are made up of mostly heme thiolate proteins called cytochrome P450s (CYPs) that facilitate hydroxylation, reduction, and oxidation reactions to convert lipophilic compounds to more hydrophilic compounds that are more easily excreted [34, 37, 44, 45]. These enzymes metabolize a wide variety of medications, steroids, fatty acids, and procarcinogens [46]. Examples of CYPs are CYP1A2, CY2C9, CYP2C19, CYP2D6, and CYP3A4 [38]. Phase II enzymes are conjugative by taking advantage of the hydrophilic groups added by Phase I enzymes. These conjugative reactions are mainly glucuronidation, sulfation, acetylation, and methylation. Examples of these enzymes are UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and glutathione S-transferases (GSTs) [34, 37, 38]. Drug transporters are important in transporting hydrophilic drugs, metabolites, and conjugated metabolites across cell membranes and facilitating their elimination in the bile and urine. Drug transporters are also important in mediating delivery of drugs or their metabolites to their therapeutic target. Important examples of drug transporters are P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), organic anion transporters (OATs), organic cation transporters (OCTs), and organic anion transporting polypeptides (OATPs) [34, 47, 48].

Genetic variation in drug-metabolizing enzymes and drug transporters are of key importance in pharmacogenomics. A variety of genetic modifications can cause alterations to these proteins, including gene deletions and duplications, known as copy number variation (CNV), insertions and deletions, as well as point mutations,

known as single nucleotide polymorphisms (SNPs). A polymorphism is defined as a genetic mutation that is present at a frequency of greater than 1% [34]. Genetic variation can occur in coding (exons) or noncoding (introns, 5'UTR and 3'UTR) regions [3].

1.B. Cytochrome P450s

The cytochrome P450 family is the most important class of enzymes in overall drug metabolism, accounting for 78% of metabolism of drugs cleared through the liver [49]. The Human Genome Project has identified 57 active enzymes and 58 pseudogenes [37, 50-52]. CYPs are a superfamily of hemeproteins, which are found in the smooth endoplasmic reticulum (ER) membrane. These enzymes have two main roles: metabolize exogenous drugs and xenobiotics as well as metabolize hormones, vitamins, and fatty acids [53]. They are expressed in several extrahepatic tissues including the intestine, lung, kidney, and brain but are preferentially expressed in the centrilobular area of the liver [34, 37, 54]. "Cyto" refers to microsomal vesicles while "chrome" refers to color. The "P" alludes to the pigmentation, red, conferred by the heme, and 450 refers to the maximum absorbance of 450 nm when the enzyme is bound to carbon monoxide [8, 36, 37, 55, 56].

There are 16 human families of CYPs [33, 34, 36, 55]. A family is defined as enzymes that have ≥40% amino acid identity and are designated by a number. Families are divided into subfamilies that have ≥ 55% amino acid identity and are designated by a number. Finally, numbers are given to designate different genes

[36, 37, 55, 57]. There are 3 major groups of CYPs: 1) CYP1-3 families have lower affinity for substrates and are less conserved but are responsible for 70-80% of all Phase I metabolism 2) CYP4 family metabolizes fatty acids and some xenobiotics and 3) CYP5-51 families have high affinity for endogenous substrates and are relatively well conserved [34, 37].

1.B.i. CYP Evolution

The first cytochrome is believed to have arisen less than 3.5 billion years ago and to have functioned anaerobically [58, 59]. Since then, CYPs have undergone multiple rounds of expansion facilitated by gene duplication. The first round occurred around 1.5 billion years ago. This expansion gave rise to CYPs that metabolized fatty acids and cholesterol [60]. The next expansion occurred around 900 million years ago. This resulted in CYPs that metabolize steroids. One of these CYPs later gave rise to current day CYP families 1 and 2. Finally, 400 million years ago, CYPs underwent another round of expansion. This resulted in several CYP families that metabolize xenobiotics. This last expansion is thought to be driven by aquatic organisms vast change in diet upon introduction onto land as well as terrestrial organisms introduction to combustion products [61, 62].

Despite multiple distinct rounds of expansion, most CYP families are continuously changing. Gene duplication allows for one copy to diverge while the other copy maintains its original function. This often creates a pseudogene,

however it sometimes increases the overall fitness of the organism. For example, CYP often arose by metabolizing toxic substances from organisms' diets [62]. Because CYPs are beneficial in order to process a wide variety of changing toxins, they have become a rapidly evolving gene. Change in a gene is measured by length of time for a unit of evolutionary period. CYPs unit of evolutionary period is about 2 – 4 million years. However, histones unit of evolutionary period is about 400 million years while immunoglobulins are about 700,000 years [61, 62].

1.B.ii. CYP Structure and Conserved Regions

Most CYPs have around 480 to 560 amino acids. They can be categorized into three groups based on location: 1) ER membrane (microsomal-type) 2) mitochondrial membrane (mitochondrial-type) or 3) cytosol (rare in eukaryotes) [57]. Microsomal CYPs are differentiated by their signal-anchor sequence, located at the N-terminal, which targets the enzyme to the endoplasmic reticulum membrane. This signal domain is made up of 20-25 hydrophobic residues and is distinguished by charged residues on either side, basic residues toward the C-terminal and negative residues near the N-terminal. These charged residues ensure that the CYP is properly inserted into the membrane, luminal side of the ER with enzyme exposed to cytoplasm. The hydrophobic region serves as the stop-transfer sequence [57, 63-65]. However, mitochondrial CYPs have a mitochondria-targeted sequence instead. This sequence acts to stall the folding

of the catalytic site until enzyme is transported into the mitochondria where it is then cleaved [57].

Although the CYP superfamily share only 20% sequence identity, they do share overall folding and topology [66]. They have an alpha helix rich carboxy-terminal and a beta-sheet rich amino-terminal [46]. Parallel helices (D, L, and I) as well as antiparallel helix (E) make up the common structure [67]. The heme binds between helix I and L to the Cys-heme-ligand loop containing the sequence, FxxGx(H/R)xCxG; the cysteine is essential as if forms the fifth ligand to the heme [68, 69]. Helix I is located near the center of the enzyme and within the heme pocket. It contains the conserved sequence (G/A)Gx(D/E)T. The threonine residue is part of the oxygen-binding pocket and is involved in catalysis [70-72]. Another conserved sequence, EXXR, is located in helix K. This sequence is key for enzyme function [69].

There are six substrate recognition sites located within helices F, G, and I [73]. These sites affect substrate specificity and are flexible to accommodate better substrate binding [74]. Any genetic changes that alter amino acid residues within regions can cause changes in drug affinities and metabolism [73].

1.B.iii. CYP Biochemistry and Catalytic Cycle

CYPs oxidize various toxins, medications, and endogenous substrates. Mechanistically, these enzymes split molecular oxygen to incorporate one as a

functional group on the substrate while the other is released as a water molecule. This reaction requires an electron donor, the reducing agent NADPH. The general formula is NADPH + O_2 + SH + H^+ -> NAD(P)$^+$ + SOH + H_2O [46]. Figure 1.1 illustrates the general CYP catalytic cycle. CYPs remain in an unreactive state until binding of the substrate. The first step is a transfer of an electron from cytochrome P450 reductase to reduce the iron from 3+ to 2+ charge. Next, the CYP complex binds molecular oxygen and triggers another electron transfer from either cytochrome P450 reductase or cytochrome b5. Two protons are accepted and the iron returns to a 3+ state. Finally, an oxygen atom is transferred to the substrate. The oxidized substrate is then released [75, 76]. There are three abortive steps (Figure 1.1), called uncoupling, within this cycle that return the enzyme to its resting state. Each produce either a superoxide anion, hydrogen peroxide, or water and occur at different intermediate states [77].

1.B.iv. CYP Regulation

There are four different types of CYP regulation: xenobiotic-inducible [78], sex-specific, tissue-specific [79], and developmental [80] regulation. CYP induction is an important mechanism in protecting an organism from changing toxins. For example, phenobarbital is a known broad CYP inducer [78, 81]. There are three receptors present in the cytosol that detect toxins. The pregnane X-receptor (PXR) regulates CYP2C9 and CYP3A4 [82-85], the aryl hydrocarbon receptor (AhR) regulates CYP1A1 and CYP1A2 [86], while constitutive androgen receptor (CAR) regulates CYP2C9 and CYP3A4 [82-85]. There have been

polymorphisms reported in all three of these receptors that effect CYP expression [87]. CYPs are also regulated in a sex-specific manner through the endocrine system and gonadal hormones. The endocrine system also plays a role in tissue-specific regulation [88].

1.C. Academic-Community Research Partnership with the Confederated Salish and Kootenai Tribes

The Confederated Salish and Kootenai Tribes (CSKT) are located in northwest Montana on the Flathead Indian Reservation. Three tribes reside on this reservation as part of CSKT: the Bitterroot Salish, Upper Pend d'Oreille, and Kootenai. Our laboratory is involved in a research partnership with CSKT to explore the use of pharmacogenomics within the tribe. With personalized medicine on the rise, some populations with health disparities are not always included in research, and therefore, do not benefit from gene-guided therapies. There is little known about pharmacogenomic variation within understudied populations, like American Indian populations. Because allele frequencies are diverse among world populations, allele frequencies within American Indians cannot be assumed to be similar to other studied populations [89, 90]. It is important to investigate frequencies of genetic variants in all populations in order to utilize pharmacogenomic testing.

Members of our laboratory have met with Tribal Council, Tribal Health, and a community advisory board to assure there is proper approval before any

research began. The community stated their main interest was in anticancer pharmacogenomics, mainly tamoxifen. CYP2C9, CYP2D6, CYP3A4, and CYP3A5 are responsible for 75% of all phase I drug metabolism, including several other anticancer agents. Therefore, our laboratory analyzed blood samples from tribal volunteers to explore the genetic variation with *CYP2C9*, *CYP2D6*, *CYP3A4*, and *CYP3A5*. Research participants, 18 years and older, were recruited at powwows, health fairs and career fairs and were asked tribal affiliation and blood quanta. DNA was extracted from whole blood and *CYP2D6* (entire gene) was resequenced in 187 participants, while *CYP2C9*, *CYP3A4*, and *CYP3A5* (exons and flanking intron regions) was resequenced in 94 random participants [90].

1.C.i. *CYP2C9* Resequencing

CYPC9 makes up 20% of hepatic CYP content and metabolizes about 15% of medications currently on the market, several with narrow therapeutic windows. Table 1.1 lists common substrates, inducers, and inhibitors for CYP2C9. Examples of substrates are warfarin, ibuprofen, and phenytoin [3, 36, 37, 46]. There are two important variants of *CYP2C9*, *CYP2C9*2* and *CYP2C9*3* (Table 1.2) that can cause large interindividual variability as well as cause adverse events [91, 92]. Together, these alleles are in seen in about 18% of European descendants, but much less so in other populations (Table 1.3). *CYP2C9*2* and *CYP2C9*3* encode for proteins with reduced intrinsic clearance. This effect is substrate specific but can caused reduced activity up to 90% [92, 93].

In resequencing *CYP2C9* in the CSKT population, our laboratory found 41 SNPs, 11 novel (most with very low frequencies). Also *CYP2C9*2* and *CYP2C9*3* were found to at a frequency lower than those found in European descendants. These SNPs cause decrease function but, due to their low frequencies, may play a minor role in *CYP2C9* interindividual variability in the CSKT. Also, low level of linkage was seen between *CYP2C9* SNPs. Table 1.3 lists the allele frequencies of interesting SNPs identified in the CSKT population.

1.C.ii. *CYP2D6* Resequencing

CYP2D6 is another important drug-metabolizing enzyme in the CYP2 family. There are several polymorphisms of *CYP2D6* that can cause a large clinical impact (Table 1.4). Although it only makes up two percent of total hepatic CYP content, it takes part in the metabolism of 15% of drugs on the market. CYP2D6 metabolizes several different substrates: propranolol, paroxetine, trazodone, codeine, and fentanyl (Table 1.1) [46, 49]. CYP2D6 is the only non-inducible CYP, so genetic variation accounts for much of the interindividual variability [49, 94]. There are more than 80 known variants, which could drastically change metabolism [95]. PMs are more common in Caucasians with 5-10% expressing a null allele. However, only 0-1% of Africans and Asians are classified as PMs. The most common allele responsible for the PM phenotype is *CYP2D6*4*. IMs are more common in Asians with 50% expressing the *CYP2D6*10* allele. Only, 10-15% of Caucasians are classified as IMs, expressing the *CYP2D6*41* allele,

encoding for a SNP that causes a fraction to missplice, and 30% of Africans express the *CYP2D6*17* allele. UMs are more common in African populations. The frequency of gene duplications is present in up to 50% in some populations and can cause up to 30-fold higher amounts of metabolite [94, 96]. It is thought that gene duplications evolved as a result of dietary pressure [94].

Upon resequencing *CYP2D6* in the CSKT population, our lab found 76 SNPs with 9 identified as novel. Individuals with multiple copies of *CYP2D6* were found to be low in this population (1.34% of alleles). The major SNPs were found to be at similar frequencies found in Caucasians (Table 1.5); 1.1% are UM, 87.2% are EM, 3.2% are IM, and 5.9% are PM. However, there was a high level of linkage seen between *CYP2D6* SNPs, including several novel haplotypes identified. The functional consequences of these haplotypes are unclear.

1.C.iii. *CYP3A4* and *CYP3A5* Resequencing

CYP3A4 is the highest expressed CYP in the liver and intestine, making up to 60% of total hepatic CYP expression [97]. Also, its presence in the small intestine is a large factor of first-pass effect [98]. CYP3A4 metabolizes a large range of substrates, totaling more than 120 different medications (Table 1.1), such as midazolam, saquinavir, erythromycin, diazepam, verapamil, tacrolimus, and simvastatin. CYP3A4 also metabolizes procarcinogens and endogenous substrates like testosterone and progesterone [99].

Most variants within *CYP3A4* occur at a frequency of less than 5% and rarely occur has homozygotes, [30, 32, 100-105] although, not all have been well characterized. Figure 2.2 shows a map of the exons and introns of *CYP3A4* with relative locations of major SNPs.

*CYP3A4*1B* has been identified in the 5'UTR, however, there is confounding data as to its clinical effect. Some investigators report this SNP causes decreased nuclear protein binding [106] and its presence has been linked to different diseases such as prostate cancer [107, 108]. However, *in vivo* and *in vitro* data using probe drugs are not so clear. Using a luciferase expression assay, investigators report an increased rate of expression for the *CYP3A4*1B* allele [109, 110]. Although not significant, it has also been reported that human livers expressing this SNP have an increased rate of nifedipine metabolism. However, other *in vitro* and *in vivo* studies show no such association between genotype and phenotype [101, 111, 112].

Coding SNPs seem to be more localized within exons 5-7 and 11-12 and frequencies are reported to be low (<5%). Most SNPs result in minimal, if any, change in drug metabolism. Those that do change enzyme activity, appear to do so in a substrate dependent fashion. *CYP3A4*2* causes decreased clearance and a 6-fold increase in the K_m in nifedipine metabolism, however, no change in the metabolism rate of testosterone [102, 113]. *CYP3A4*3* encodes for a SNP within the heme-binding pocket, although, no change in clearance is observed

[103, 104, 114]. *CYP3A4*8* and *CYP3A4*13* are reported to have low protein content in a cells and are expected to cause decreased levels *in vivo* [103, 104]. *CYP3A4*12* results in an increased clearance of testosterone 15β and 2β-hydroxylation [103]. *CYP3A4*17* is reported to cause a reduction in testosterone metabolism while *CYP3A4*18* causes an increase in testosterone metabolism [104].

Intronic SNPs are more rare and are mostly present at a frequency of less than 1%, however, a few are reported at much higher frequencies. G20338A and T15871C are present at approximately 50% in African American and 6.5% in Caucasians. Interestingly, these two alleles are commonly inherited together in African Americans, although no clinical significance has been reported [100]. *CYP3A4*1G* is another SNP, located in intron 10, seen at higher frequencies in various populations [115]. There is mixed data, although, most suggest decreased clearance [116-120]. *CYP3A4*22*, located in intron 6, is found in Caucasian populations [121, 122]. Again, this SNP has mixed data, however, most suggest it leads to decreased clearance [122-126].

Resequencing *CYP3A4* in CSKT populations resulted in identification of 15 SNPs, of which 4 are novel SNPs. Major SNPs identified are listed in Table 1.6. All SNPs were seen with frequencies similar to Caucasians, except for *CYP3A4*1G*, seen with a frequency similar to Japanese and Chinese populations (26.8%) (Table 1.7). This results in 7% of individuals with the homozygous,

*CYP3A4*1G/*1G* genotype, and 39% as heterozygous for *1G. This SNP has conflicting data as to its clinical relevance and will be discussed in section 1.C.v.

CYP3A5 is expressed in the liver, and is the only CYP3A isoform expressed outside the liver and intestine tissues, such as the kidney [127], prostate [128], and lung [129, 130]. CYP3A5 has a similar structure to CYP3A4 and metabolizes many of the same substrates, however, it usually does so with slower turnover rates [131, 132]. Because CYP3A4 and CYP3A5 have similar substrates, there is no known specific substrate of CYP3A5. This makes it difficult to measure CYP3A5 specific activity [129].

Polymorphisms with the largest clinical impact can be divided into coding region variants and intronic SNPs that cause frameshifts or splicing defects [129]. Figure 2.2 shows a map of introns and exons and the location of the major SNPs. SNPs have been reported within the 5' untranslated region, *CYP3A5*1B* and CYP3A5**1C*, however, they are seen to have no clinical significance [100, 133]. *CYP3A5*6, *7, *8, *9,* and **10* are all SNPs within the coding region that result in change in enzyme function. *CYP3A5*6* results in a truncated, nonfunctional protein [100, 129]. *CYP3A5*7* actually encodes for a base insertion that causes a frameshift, and again, a nonfunctional protein [129, 133, 134]. *CYP3A5*8* and *CYP3A5*9* encode for amino acid changes that result in roughly 50% enzyme activity. *CYP3A5*10* results in an inactive enzyme through an amino acid change in the heme-binding region [129].

*CYP3A5*3* and *CYP3A5*5* are clinically important intronic SNPs [129]. *CYP3A5*3* is the most common polymorphism seen in *CYP3A5*. This intronic SNP causes a splice variant with integration of a portion of intron 3. This leads to a frameshift and premature termination. This deleterious SNP is more commonly found in Caucasians and Asians, however, Africans more often express the functional, wild-type, enzyme [100, 133]. *CYP3A5*5* causes a change in a base in the splicing donor site and results in truncated protein [129, 134].

Resequencing of *CYP3A5* in CSKT populations resulted in identification of 10 SNPs; 1 was novel and found at a low frequency. Other major identified SNPs are listed in Table 1.8. *CYP3A5*3* was found at frequency of 92.47%, similar to Caucasians (Table 1.9). This results in 86% of the CSKT population as homozygous for *CYP3A5*3*, 14% are heterozygous individuals, and zero were homozygous for *CYP3A5*1* (wild-type).

Overall, *CYP3A4* and *CYP3A5* exhibit a high level of linkage. However, there was a break in the linkage between *CYP3A4*1G* and *CYP3A5*1* (0.158 LD). The clinical relevance of this linkage will be explored in following section, Implications.

1.C.iv. Implications

Despite novel SNPs found in *CYP2C9*, *CYP2D6*, *CYP3A4*, and *CYP3A5*, they are not expected to play a large role in interindividual variability because of their

low frequencies. Most common allele frequencies were found to be similar to those found in Caucasians. Although, one SNP, *CYP3A4*1G*, was found at a much higher frequency. *CYP3A4*1G* frequency is much more similar to Japanese and Chinese populations. However, unlike all other populations where *CYP3A4*1G* is found in high linkage with *CYP3A5*1*, CSKT has a novel break in the linkage between *CYP3A4*1G* and *CYP3A5*1*. In combination, the relatively common *CYP3A4*1G* (26.81% allele frequency) and the high frequency *CYP3A5*3* (92.47% allele frequency) in the CSKT could have large clinical implications because *CYP3A4*1G* data suggest lower activity [116-120] and *CYP3A5*3* encodes for a nonfunctional protein [100, 133]. Individuals carrying *CYP3A4*1G* and *CYP3A5*3* could have severely diminished CYP3A activity.

1.C.v. *CYP3A4*1G* Data

*CYP3A4*1G* is an intronic SNP found within intron 10 [135]. Again, most data suggest diminished activity for *CYP3A4*1G*. There have been several studies investigating the effect of *CYP3A4*1G* on fentanyl consumption post gynecological surgery [116, 117, 119, 120]. Zhang *et al.* found a trend of decreased fentanyl consumption for CYP3A4*1G carriers [119], while Dong *et al.* found a statistical difference of decreased fentanyl consumption between only *CYP3A4*1G/*1G* status with both the heterozygote and wild-type [117]. Zhang *et al.*, interestingly, found a significant decrease in fentanyl consumption for patients carrying both *CYP3A4*1G/*1G* and *CYP3A5*3/*3* [116]. This haplotype is seen in high frequency within the CSKT and could change their drug metabolism. More

recently, Yuan *et al.* analyzed the effect of *CYP3A4*1G* on fentanyl consumption as well as plasma concentrations [120]. They found that patients who expressed *CYP3A4*1G/*1G* had statistically higher fentanyl plasma concentrations and required lower fentanyl doses than those who expressed heterozygote or wild-type genotypes. All patients in all studies were female; *CYP3A4* genotype could have a higher impact on females, because they are reported to express more CYP3A4 [136]. With higher metabolism rates due to more protein content, changes in activity can results in larger changes in metabolism rates.

A trend of decreasing function of *CYP3A4*1G* was found when investigating its effect on atorvastatin efficacy [118]. A gene-dose effect was found on the mean reduction of serum total cholesterol after atorvastatin treatment. However, this effect was not seen after simvastatin treatment, suggesting potential substrate-specific effects.

There are also a few studies that suggest *CYP3A4*1G* is a gain-of-function SNP. Miura *et al.* found that tacrolimus pharmacokinetics were significantly altered in patients carrying *CYP3A4*1G* and were CYP3A5 expressers; these patients had lower exposures and initial concentrations [137]. Zuo *et al.* also found this same effect. Patients carrying *CYP3A4*1G* and *CYP3A5*1* had the highest tacrolimus clearance than other haplotypes. Also*, CYP3A4*1G* carriers and CYP3A5 nonexpressers also had higher clearance than those expressing *CYP3A4*1/*1* and *CYP3A5*3/*3* [138]. He *et al.* investigated the effect of *CYP3A4*1G* on

coronary heart disease (CHD) risk. Using a multivariate regression, they found that those who express *CYP3A4*1G/*1G* are at increased risk of CHD. The authors suggest that *CYP3A4*1G* is a gain of function. They argue that because CYP3A4 metabolizes estrogen, and estrogen is protective against CHD, that those who carry *CYP3A4*1G* must metabolize estrogen more quickly [139]. However, no probe drugs were used to actually assay *CYP3A4*1G* clearance. Another study, looking at *CYP3A4*1G* effect on risperidone metabolism, found no significant difference between plasma risperidone concentrations [140]. However, those expressing *CYP3A4*1G/*1G* did have much lower plasma levels. This study performed a Kruskal-Wallis analysis between all three genotypes groups. As in other studies, perhaps they would have found significance if they had grouped *CYP3A4*1/*1* with *CYP3A4*1/*1G* and analyzed their plasma concentrations with *CYP3A4*1G/*1G*. Because risperidone is mainly metabolized by CYP2D6, the authors suggest that changes in CYP3A4 function would be of greater impact in those who are CYP2D6 poor metabolizers. *CYP2D6* was not genotyped, which again, may have resulting in *CYP3A4*1G* significance when *CYP2D6* genotype was accounted for.

The combined data suggest a possible substrate-dependent effect of *CYP3A4*1G*. However, most data do indicate *CYP3A4*1G* results in decreased clearance. More research needs to be done to identify the effect of *CYP3A4*1G* as well as when inherited with *CYP3A5*3/*3*.

1.D. CYP3A Subfamily

The CYP3A subfamily comprises four functional genes, CYPs 3A4, 3A5, 3A7, and 3A43, as well as two pseudogenes [129, 141]. All six genes are located inline with one another on chromosome 7 [129]. This subfamily shares many substrates but differ in tissue expression [34, 46, 129]. CYP3A4 metabolizes around 50% of drugs currently on the market and could, therefore, be considered one of the most important drug metabolizing enzymes (Table 1.1) [3, 34, 36, 46, 129, 142-144]. Common SNPs in *CYP3A5* encode for deleterious protein and, therefore, is variably expressed. CYP3A7 is expressed in fetal livers up until about 6 months of age. However, 10% of adult livers continue to express CYP3A7 and can contribute up to almost a quarter of total CYP3A content, which can contribute to clearance [145-147]. CYP3A7 expression into adulthood exists more in Japanese populations, with 3A7 accounting for up to 40% of 3A content [147]. CYP3A43 has been found in several tissues, however, at very low quantities. Also, it exhibits reduced activity towards testosterone, so it is not expected to play much of a role in xenobiotic metabolism [148].

1.D.i. *CYP3A4*

The *CYP3A4* gene is 27kb and includes 13 exons and 12 introns. The gene encodes for a 57 kDa protein made up of 502 amino acids [57, 89, 149, 150]. Substrates of CYP3A4 are large and lipophilic [49]. CYP3A4 can metabolize a wide range of structural substrates due to its large and flexible binding pocket

[151, 152]. It has been known to bind multiple substrates at once which can cause increased or decreased product formation [153].

1.D.ii. *CYP3A4* Variability

It has been estimated that 90% of *CYP3A4* interindividual variation is due to genetic factors [154]. Variability in CYP3A4 expression can cause a dramatic clinical effect due to the large number of substrates [30]. There is large interindividual variability seen in CYP3A4 expression, up to a 40-fold change [112, 155, 156]. However, most populations tend to lie within a 4- to 6-fold variation [106, 156-158]. Genetic variants do not account for all the variability, however, there are numerous factors that need to be considered [34, 129]. There have been over 20 variants reported that can explain for some variation. Also, CYP3A4 can be induced, through increased transcription, by certain xenobiotics, such as rifampicin, barbiturates, carbamazepine, glucocorticoids, and St. John's Wort [34, 36, 46, 159]. CYP3A4 can also be inhibited by various xenobiotics. Ketoconazole, saquinavir, fluoxetine, and grapefruit juice have been reported to as inhibitors. Potent inhibitors can cause plasma levels of the drug to increases 20-fold [34, 36, 160]. *CYP3A4* is the only P450 that is expressed at different levels between sexes, with women expresses up to 2-fold more protein than men [136]. Finally, some interindividual variation may exist due to hormonal regulation of CYPs. Hormones can be endogenously circulating or present in diet [28]. All these factors can make determining genotype-phenotype correlations difficult.

1.D.iii. *CYP3A4* Phenotypes

Phenotypes seen from variation in *CYP3A4* are unimodal, unlike what is seen in *CYP2D6*. *In vivo* treatment with midazolam resulted in outliers exhibiting higher clearances [158]. However, when treated with nifedipine, outliers were present with lower clearances [161]. This unimodal distribution suggests that no single factor can be used to predict *CYP3A4* phenotype [30].

1.D.iv. *CYP3A5*

CYP3A5 has 13 exons and is made up of 502 amino acids [32, 162]. Unlike CYP3A4, CYP3A5 is not markedly induced [163]. However, *CYP3A5* is polymorphic with the most common SNP encoding for deleterious protein [164].

1.D.v. *CYP3A5* Phenotypes

In individuals who express wild-type CYP3A5 enzyme, CYP3A5 can make up 50% of CYP3A content [100]. This can result in large interindividual variability in CYP3A metabolism; those carrying the *CYP3A5*3/*3* genotype metabolize midazolam at less than half the rate of those carrying at least one *CYP3A5*1* functional allele [100, 156]. Due to the dramatic decrease in activity, *CYP3A5* genotype has been correlated with statin treatment side effects[165].

1.D.vi. *CYP3A4* and *CYP3A5* Linkage Disequilibrium

CYP3A4 and *CYP3A5* are found in a high degree of linkage disequilibrium in Caucasian and Asian populations with the most common haplotype being

*CYP3A4*1 and CYP3A5*3* [3]. Caucasians also exhibit linkage disequilibrium between *CYP3A4*1B* and *CYP3A5*1* [100, 106]. It is hypothesized that the two haplotypes could result in similar activity; the two alleles compensate for one another. African populations have very diverse haplotypes with no significant degree of linkage disequilibrium seen [3], however, they are more likely to carry both the *CYP3A4*1B* and the *CYP3A5*1* alleles [100, 166].

1.E. Specific Aims

The objective of this project is to determine the functional consequence of the *CYP3A4*1G* genetic variant using *in vitro* methods. The specific goals of Aim 1 was to use immortalized human lymphocytes with differing *CYP3A4* genotypes (*CYP3A4*1/*1, CYP3A4*1/*1G, and CYP3A4*1G/*1G*) to analyze the effect of *CYP3A4*1G* on relative mRNA content, protein content, and enzyme activity. Aim 2 used human liver microsomes to, again, determine the effect of *CYP3A4*1G* on protein content and enzyme activity.

Table 1.1. List of CYP2C9, CYP2D6, and CYP3A4/5 Common Substrates, Inhibitors, and Inducers

	Substrates	Inhibitors	Inducers
CYP2C9	ibuprofen phenytoin tolbutaminde S-warfarin	amiodarone fluconazole fluvoxamine fluoxetine sulfaphenazole	rifampin
CYP2D6	atomexetine bufuralol debrisoquine desipramine dextromethorphan	bupropion fluoxetine paroxetine quinidine	
CYP3A4/CYP3A5	buspirone erythromycin felodipine dextromethorphan lovastatin midazolam nifedipine simvastatin terfenadine testosterone triazolam	azamulin clarithromycin erythromycin fluconazole grapefruit juice indinavir itraconazole ketoconazole ritonavir saquinavir troleandomycin verapamil	carbamazepine phenytoin rifampin St. John's wort

This list is not intended to be inclusive. Adapted from FDA website "Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers."

Table 1.2. **Major CYP2C9 Alleles**

Allele	Nucleotide Change	Protein Change	Activity Change
CYP2C9*2 (rs1799853)	430C>T	R144C	decreased
CYP2C9*3 (rs1057910)	1075C>C	I359L	decreased

Adapted from Fohner, *et al,* 2013 [90, 167, 168].

Table 1.3. CYP2C9 Allele Frequencies

Allele	CSKT	CEU	YRI	JPT	CHB	Canadian Inuit	Canadian First Nation	Central America (Tepehuano/ Mestizos)
rs4918758	25.0	35.8	30.1	41.3	33.7			
CYP2C9*2	5.17	10.4	0	0	0	0	3.0	1 - 7
CYP2C9*3	2.69	5.8	0	2.3	4.7	0	6.0	1.5
rs28371689	30.77							
rs1057911	2.7	5.8	0	3.4	4.4			

CSKT = Confederated Salish and Kootenai Tribes (n=188 chromosomes). HapMap populations: CEU = Utah residents with ancestry from northern and western Europe (n=120 chromosomes); YRI = Yoruba in Ibadan, Nigeria (n=120 chromosomes); JPT = Japanese in Tokyo, Japan (n=90 chromosomes); CHB = Han Chinese in Beijing, China (n=90 chromosomes). Adapted from Fohner, *et al.,* 2013 [90].

Table 1.4. Major CYP2D6 Alleles

Allele	Nucleotide Changes	Protein Effect	Phenotype
CYP2D6*1	Wild-type	None	EM
CYP2D6*2	2850C>T; 4180G>C	R296C; S486T	
CYP2D6*3	2549delA	Frameshift	PM
CYP2D6*4	100C>T; 1846G>A; 4180 G>C	P34S; splicing defect; S486T	PM
CYP2D6*5	Gene Deletion	Gene deletion	PM
CYP2D6*9	2615delAAG	K281del	IM
CYP2D6*10	100C>T; 4180G>C	P34S; S486T	IM
CYP2D6*17	1023C>T; 2850C>T	Amino Acid Substitution	IM
CYP2D6*28	19G>A; 1704C>G; 2850C>T; 4180G>C	V7M; Q151E; R296C; S486T	ND
CYP2D6*33	2483G>T	A237S	EM
CYP2D6*35	31G>A; 2850C>T; 4180G>C	V11M; R296C; S486T	EM
CYP2D6*41	2850C>T; 2988G>A; 4180G>C	R296C; splicing defect; S486T	IM

Phenotypes denoted as: PM = Poor Metabolizer; IM = Intermediate Metabolizer.
Adapted from Fohner, *et al.,* 2013 [90].

Table 1.5. Major CYP2D6 Allele Frequencies

Allele	CSKT	European	African American	Japanese	Chinese	Canadian Inuit	Canadian First Nation	Central America (Tepehuano/ Mestizos)	South America (Embera/ Mapuche)
CYP2D6*1	37.57	28.6- 83.0	29.7 - 83.0	27.0 - 93.8	18.0 - 38.4	89.10	68.0 - 94.0	38.8 - 99.4	39.9 - 84.9
CYP2D6*3	0.27	0 - 3.20	0.18 - 0.60	0	0	0	0	0 - 1.44	0
CYP2D6*4	20.86	11.3- 33.4	3.86 - 7.8	0 - 0.77	0 - 1.10	6.7 - 8.3	3.0	0.6 - 19.4	3.6 - 17.8
CYP2D6*5	1.34	0 - 6.9	2.80 - 6.90	4.1 - 7.2	2.54 - 9.60			0.80 - 4.60	0 - 4.20
CYP2D6*9	0.80	0 - 3.8	0.18 - 1.15	0	0 - 1.27				0
CYP2D6*10	1.34	0.9 - 8.0	2.70 - 7.50	8.6 - 45.9	22.4 - 64.1	2.30	3.0	0 - 12.45	1.80 - 7.10
CYP2D6*17	0	0 - 1.11	13.7 - 26.0	0	0 - 0.21			0 - 10.20	
CYP2D6*28	0.27	0		0					
CYP2D6*33	0.53			0					
CYP2D6*35	1.07	4.8 - 8.5	0.38 - 1.10	0	0				0.85
CYP2D6*41	11.23	6.9 - 14.0	1.84 - 14.9	0.51 - 2.60	2.2 - 4.0			2.54	

CSKT = Confederated Salish and Kootenai Tribes (n=374 chromosomes). Adapted from Fohner, *et al.,* 2013 [90].

Table 1.6. Major CYP3A4 alleles

Allele	Nucleotide Change	Protein Effect	Change in Activity
CYP3A4*1B (rs2740574)	-392A<G	promoter	decreased
CYP3A4*1G (rs2242480)	20230G>A	intronic	decreased
CYP3A4*2 (rs55785340)	15713T>C	Ser222Pro	decreased
CYP3A4*3 (rs4986910)	23171T>C	Met445Thr	none
CYP3A4*8 (rs72552799)	13908G>A	Arg130Gln	decreased
CYP3A4*12 (rs12721629)	21896C>T	Leu373Phe	both
CYP3A4*13 (rs4986909)	22026C>T	Pro416Leu	decreased
CYP3A4*15A (rs4986907)	14269G>A	Arg162Gln	nonfunctional
CYP3A4*17 (rs4987161)	15615T>C	Phe189Ser	both
CYP3A4*18 (rs28371759)	20070T>C	Leu293Pro	both
CYP3A4*22 (rs35599367)	15389C>T	intronic	decreased

Adapted from Fohner, *et al,* 2013 [90, 135, 168].

Table 1.7. Major CYP3A4 Allele Frequencies

Allele	CSKT	CEU	YRI	JPT	CHB	Central America (Tepehuano/ Mestizos)
CYP3A4*1B	2.20	3.0	72.0	0	0.3	8.0 – 8.8
CYP3A4*1G	26.81	8.3	88.9	29.7	28.0	
CYP3A*2	0	0	0	0	0	
CYP3A4*3	0	1.2	0	0	0	
CYP3A4*8	0	0	0	0	0	
CYP3A4*12	0	0	0	0	0	
CYP3A4*13	0.60	0.4	0	0.6	1.2	
CYP3A4*15A	0.68	0	2.84	0	0	
CYP3A4*17	0	0	0	0	0	
CYP3A4*18	0	0	0	1.7	0	
CYP3A4*22	2.44	5.29	0	0	0	
rs2687116	2.27	1.8	74.4	0	0	

CSKT = Confederated Salish and Kootenai Tribes (n=188 chromosomes). HapMap populations: CEU = Utah residents with ancestry from northern and western Europe (n=120 chromosomes); YRI = Yoruba in Ibadan, Nigeria (n=120 chromosomes); JPT = Japanese in Tokyo, Japan (n=90 chromosomes); CHB = Han Chinese in Beijing, China (n=90 chromosomes). Adapted from Fohner, *et al.,* 2013 [90, 121, 169].

Table 1.8. Major CYP3A5 Alleles

Allele	Nucleotide Change	Protein Effect	Change in Activity
CYP3A5*3 (rs776746)	6986A>G	intronic	nonfunctional
CYP3A5*6 (rs10264272)	14690G>A	truncation	nonfunctional
CYP3A5*7 (rs76293380)	27131_27132insT	frameshift	nonfunctional
rs15524	31611T>C	3' UTR	

Adapted from Fohner, *et al,* 2013 [90, 135, 168].

Table 1.9. Major CYP3A5 Allele Frequencies

Allele	CSKT	CEU	YRI	JPT	CHB
CYP3A5*3	92.27	94.1	15.0	73.3	66.3
CYP3A5*6	0	0	16.8	0.6	1.2
CYP3A5*7	0	0	0	0	0
rs15524	10.64	4.0	72.6	28.5	33.7

CSKT = Confederated Salish and Kootenai Tribes (n=188 chromosomes). HapMap populations: CEU = Utah residents with ancestry from northern and western Europe (n=120 chromosomes); YRI = Yoruba in Ibadan, Nigeria (n=120 chromosomes); JPT = Japanese in Tokyo, Japan (n=90 chromosomes); CHB = Han Chinese in Beijing, China (n=90 chromosomes). Adapted from Fohner, *et al.,* 2013 [90, 121, 169].

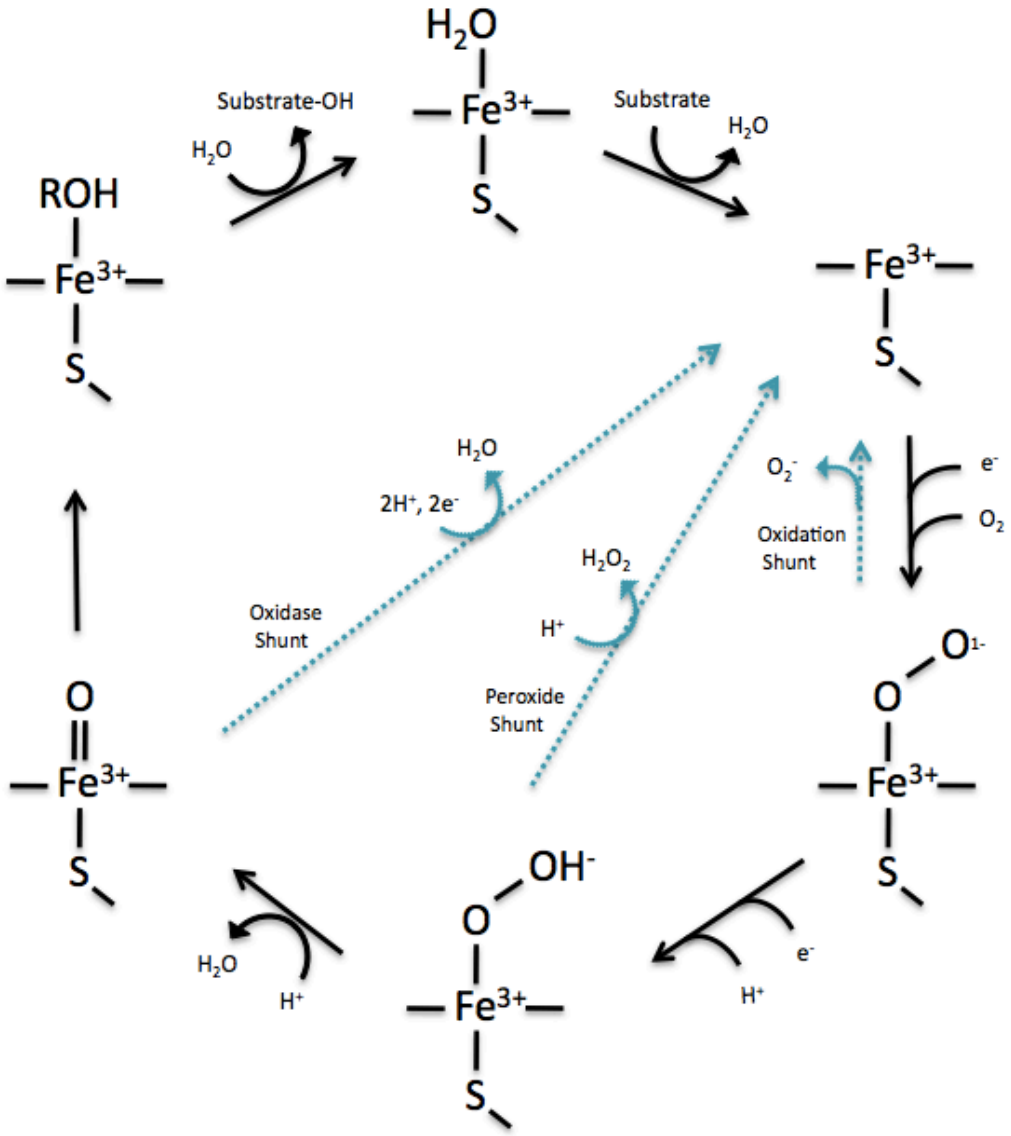
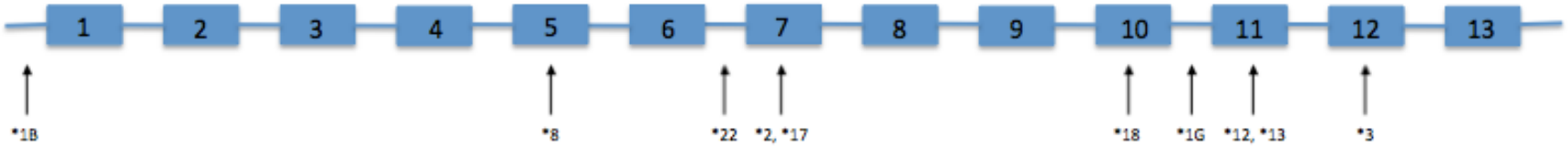


Figure 1.1. Cytochrome P450 Cycle. Adapted from [170].

A.



B.

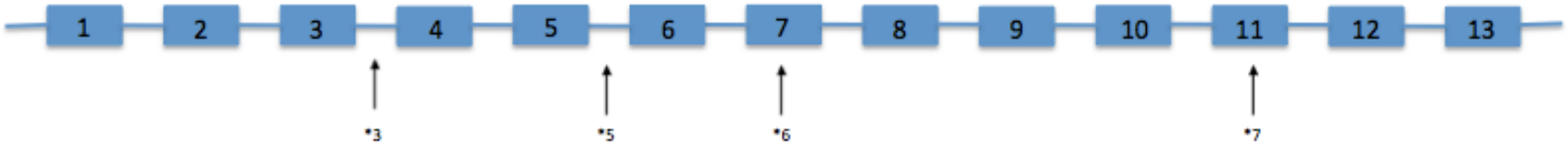


Figure 2.2. SNP Gene Map A. *CYP3A4*. **B.** *CYP3A5*. Exons are denoted by boxes and introns as lines. Adapted from Lamba, *et al.,* 2002 [30, 135, 168].

Chapter 2: Lymphocytes as Surrogates of CYP3A Drug Metabolism

2.A. Introduction

CYP3A4 is responsible for the metabolism of 50% of drugs currently on the market [129, 142, 143]. It is reported that 66 - 90% of the interindividual variability in CYP3A4 activity is a result of genetic variability [154]. While there are many identified SNPs, several do not have conclusive evidence linking them to the interindividual change [168]. *CYP3A4*1G* is an SNP located within intron 10 (20230G>A) and has conflicting reports of its impact on CYP3A4 activity. Most reports show a decrease in activity [116-120], however, some show an increase in function [137-139].

Genetic variants can be measured *in vivo*, where CYP activity is measured via administration of probe drugs or radiolabeled drugs [171, 172]. Probe drugs are metabolized by a single drug-metabolizing enzyme and are administered to identify the function of that enzyme, or the change in function of the enzyme due to the presence of a SNP. Plasma concentrations of the probe drug and metabolites are measured to estimate various pharmacokinetic parameters [172]. Also, one could measure CYP changes in activity, protein, and mRNA levels within liver hepatocytes or microsomes after performing a liver biopsy [173, 174]. However, both methods present some considerable disadvantages. Probe drug administration creates the risk of adverse events, multiple blood draws and urine collection, length of time needed to collect samples from patient, and the associated high cost [175]. Liver biopsies are not routinely performed, especially for the purpose of the phenotyping a patient [173]. These substantial

disadvantages associated with *in vivo* phenotyping presents a large barrier in clinical practice as well as research.

Because of some of the challenges associated with existing phenotyping methods, there is increased interest in using a more readily available tissue such as peripheral blood cells. These cells could be used as surrogate markers of drug metabolism or changes in drug metabolism. Peripheral blood cells are made up of red blood cells, white blood cells, and platelets. White blood cells are made in lymphoid tissues and include several subsets of cells: neutrophils, eosinophils, basophils, monocytes, and lymphocytes. Lymphocytes are responsible for recognizing foreign antigens and mounting a response [176]. There are several advantages to measuring CYP activity in lymphocytes: no need for probe drug administration, less invasive sampling, less time involved for the patient, as well as less expensive to test [175]. Several CYPs have been measured in lymphocytes, including CYP3A4 [177]. However, there are conflicting reports in literature of the consistency in measuring CYP3A4 activity and mRNA levels and its correlation to liver activity and expression [173, 175, 178-183].

The goal of this study was to determine the functional consequences of the genetic variant *CYP3A4*1G* using lymphocyte cell lines that are wild type, heterozygote, and homozygote for the *CYP3A4*1G* genotype. CYP3A4*1G functional consequence in lymphocytes was assessed by measuring 1) mRNA by

quantitative PCR, 2) protein levels by immunoblot detection, and 3) CYP3A4 activity using the substrate luciferin IPA.

2.B. Materials and Methods

2.B.i. Cells

B-lymphocytes were ordered from the National Human Genome Research Institute Sample Repository for Human Genetic Research through the Coriell Institute for Biomedical Research. Lymphocytes were selected based on *CYP3A4* and *CYP3A5* genotype*: CYP3A4*1/*1, CYP3A4*1/*1G*, and *CYP3A4*1G/*1G*; all samples were *CYP3A5*1/*1*. All samples were from females. Cells were maintained in RPMI Medium 1640 with 2mM L-glutamine and 15% FBS at 37°C and 5% CO_2. They were split to a density of 200,000 viable cells/mL every third day.

2.B.ii. RNA Isolation and cDNA synthesis

RNA was isolated using the PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA) and RNA concentration and quality was measured on a Nanodrop spectrophotometer. PureLink® DNAse Treatment (Life Technologies) was used during RNA extraction to digest any DNA. cDNA was immediately synthesized using the High Capacity RNA-to-cDNA™ Kit (Applied Biosystems®, Carlsbad, CA) on a C1000 Thermocyclyer (Bio-Rad, Hercules, CA). Samples were not frozen and immediately underwent quantitative PCR.

2.B.iii. Quantitative Real-time PCR

CYP3A4, CYP3A5, β-actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were measured using TaqMan® Gene Expression Assay (Applied Biosystems®). Primer/probe pairs were: CYPA4 Hs00604506_m1; CYP3A5 Hs01070905_m1; β-actin H99999903_m1; GAPDH Hs03929097_g1. Taqman® probes had a reporter dye, FAM™, on the 5' end and a nonfluorescent quencher (TAMRA™) on the 3' end. TaqMan® Universal Master Mix II, with UNG was used to perform amplifications. TaqMan® probe identification and amplicon length as well as exon location are defined in Table 2.1. All amplicons produced are relatively short and are not expected to PCR efficiency. Probes for *CYP3A4* and *CYP3A5* span exons, so no genomic DNA will be amplified. cDNA template, 200 ng, underwent cycling conditions of 1 cycle at 50°C for 2 min, 1 cycle of 95°C for 10 min, then 50 cycles of denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 1 min on an Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA). Each amplification was performed in quadruplicate at two separate times with independent RNA isolations. Data was normalized to housekeeping gene (ΔC_t) by subtracting its C_t from the C_t of the CYP3A4/5. C_t was defined as 10 standard deviations above average background level. Reporter signal was normalized to a passive reference dye, ROX™, included in the master mix.

2.B.iv. CYP3A4 Protein Quantitation in Lymphocytes

Protein quantitation in lymphocyte was performed by immunoblot. Total protein (10 μg) of cell lysate was added and resolved on a Tris-Hepes NH 4-20% (NuSep, Lane Cove, Australia). Protein was transferred to nitrocellulose membranes and blocked with 5% milk for 1 hour. The membrane incubated with primary anti-CYP3A4 antibody (Abnova, Taipei City, Taiwan) (1:1,000 dilution) on a rocker overnight at 4°C. Secondary anti-mouse antibody was added (1:25,000 dilution) for 1 hour at room temp. Membrane was developed using the West Femto Kit (Thermo Scientific™, Waltham, MA) and imaged on a using an LAS-3000 camera (Fujifilm, Minato, Tokyo).

2.B.v. CYP3A4 Protein Quantitation in Human Liver Microsomes

Protein quantitation in human liver microsomes was performed by immunoblot. Total protein (10 μg) were added per HLM sample and resolved on a Tris-Hepes NH 4-20% gel (NuSep) Protein was transferred to nitrocellulose membranes and blocked with 5% milk for 1 hour. The membrane incubated with primary anti-CYP3A4 antibody (Abnova) (1:1,000 dilution) on a rocker overnight at 4°C. Secondary anti-mouse antibody was added (1:25,000 dilution) for 1 hour at room temp. Membrane was developed using the West Femto Kit (Thermo Scienctific™) and imaged on an LAS-3000 camera (Fujifilm).

2.B.vi. CYP3A4 Activity in Lymphocytes

CYP3A4 activity was assessed in lymphocytes using P450-Glo™ assay system (Promega, Madison, WI). Lymphocytes were counted in serum-free media and 5 x 10^4 – 5 x 10^6 cells in suspension were placed into 1.5 ml eppendorf tubes. An equal volume of 2x P450-Glo™ substrate (Lucierin IPA) in serum free media was added to cell suspension. Tubes were incubated at 37°C and 5% CO_2. An equal volume of Luciferin Detection Reagent was added and tubes were placed on an orbital shaker for 15-20 min at room temp. Solution was transferred to a white, untreated 96-well plate and luminescence detected on a SynergyMX microplate reader (Biotek, Winooski, VT) with an integration time of 1 second/well.

2.B.vii. Data Analysis

A Tukey's post hoc test was completed to determine differences in the qPCR ΔC_ts between lymphocyte cell lines, α=0.05 (KaleidaGraph, Reading, PA).

2.C. Results

2.C.i. CYP3A4 mRNA Expression in Lymphocytes

All genes of interest were detected in all three lymphocyte cell lines, although, CYP3A4 mRNA levels were very low as demonstrated by the high C_t values (Table 2). Representative quantitative PCR traces of each cell line are shown in Figure 2.1. The C_ts of all genes are reported in Table 2.2 and ΔC_ts, *CYP3A4* and *CYP3A5* normalized to housekeeping genes, are reported in Figure 2.3. ΔC_ts show that the order of the CYP3A4 expression is as follows: CYP3A4*1/*1G >

CYP3A4*1G/*1G > CYP3A4*1/*1. There is statistical significance between all three cell lines (normalizing to β-actin: *1/*1 and *1/*1G $p<0.0001$; *1/*1 and *1G/*1G $p=0.002$; *1/*1G and *1G/*1G $p=0.031$ and normalizing to GAPDH: *1/*1 and *1/*1G $p<0.0001$; *1/*1 and *1G/*1G $p=0.045$; *1/*1G and *1G/*1G $p=0.002$). With respect to CYP3A5 expression levels, CYP3A4*1/*1G also expresses the most CYP3A5. The *1/*1G genotype expresses more CYP3A5 than the *1/*1 genotype, after normalizing to both β-actin and GAPDH, $p<0.0001$ for all comparisons. However, there is no significance between the CYP3A5 expression levels when controlling for either housekeeping gene between the *CYP3A4*1G/*1G* and *CYP3A4*1/*1* genotypes (β-actin $p=0.63$; GAPDH $p=0.4$).

To show that *CYP3A4* was amplified in qPCR, and the high C_t was not due to primer/probe degradation, the amount of cDNA added to each reaction was diluted. Instead of adding 200 ng of total RNA to each reaction, 25 ng of total RNA was added. Figure 2.3 shows the qPCR traces of the template dilution. The diluted total RNA samples have a higher C_t value indicating less CYP3A4 mRNA in the sample. Table 6 shows the C_t values of each gene for the different amounts of total RNA added as well as the ratio of C_ts for 200 ng to 25 ng. These ratios were similar across genes, confirming that the C_ts measured are not due to probe degradation.

2.C.ii. CYP3A4 Protein Expression in Lymphocytes

Immunoblots of each lymphocyte cell line were performed to quantify CYP3A4 protein content. In contrast to the qPCR data, western blot quantification (Figure 2.4) shows that *CYP3A4*1G/*1G* has the most expression, followed by *CYP3A4*1/*1G*. Wild-type *CYP3A4* had no detectable protein, however, a high molecular weight band was detected. To understand this high molecular weight band, we ran another immunoblot in human liver microsomes of the same genotypes (Figure 2.4). The high molecular weight band was not detected in any genotype of the human liver microsomes and it appears to be specific to lymphocytes.

2.C.iii. CYP3A4 Activity in Lymphocytes

Next, CYP3A4 activity in lymphocytes was measured. Cells were first incubated with 3 μM substrate for 15 min with zero activity measured. Next, time was increased to 1 hour incubation at the same substrate concentration and, again, measured zero activity. Finally, cells were treated with 25 μM substrate for 3 hours; the rate measured was negligible.

2.D. Discussion

There is interest in finding a more accessible tissue as a surrogate of drug metabolism in the liver. Blood sampling is minimally invasive, low risk, and relatively unlimited. However, CYPs are not highly expressed within lymphocytes and there are several contradicting studies as to their metabolic relevance. Our

goal was to establish whether immortalized lymphocytes, with defined *CYP3A4* and *CYP3A5* genotypes, could be used to determine the functional consequence of the *CYP3A4*1G* SNP.

Several groups have been able to quantify CYP3A4 in lymphocytes. Sempoux *et al.* and Starkel *et al*. showed that CYP3A proteins were detectable in B-lymphocytes by immunoblot and or immunohistochemistry [184, 185]. Nakamoto *et al*. were able to detect CYP3A4 mRNA in all samples of lymphocytes (n=8) by quantitative competitive (QC) RT-PCR. They were also able to measure a statistically significant level of induction after oral administration of rifampin. Along with an increase of mRNA content, this group also found an increase in the ratio 6β-hydroxycortisol to cortisol, indicating an increase in CYP3A4 liver activity. These data imply that lymphocytes can indeed be used as a surrogate for liver activity [180]. However, this group did not use primers that spanned exons, causing amplification of any DNA present in the sample. This could result in more CYP3A4 measured than is actually present as cDNA. Krovat *et al*. and Nowakowski-Gashaw *et al*. were able to detect CYP3A4 in lymphocytes using QC-PCR [182, 186]. Gashaw *et al*. was also able to measure CYP3A4 expression in all samples and also found a weak, but significant, correlation between mRNA content in lymphocytes with alprazolam clearance [173].

Some groups have been able to detect CYP3A4 but were unable to correlate levels of expression with CYP3A4 function in the liver. Finnstrom *et al*. reported

measuring CYP3A4 expression at relatively high levels. They used qPCR with 16.7 ng total RNA and a C_t cutoff value of 38, which corresponds to 1000 molecules. Most lymphocyte traces had C_ts between 33 and 37, however, they did not show housekeeping, normalizing gene traces. They were unable to find a correlation between liver and lymphocyte CYP3A4 expression [179]. Haas *et al*. also were able to measure CYP3A4 mRNA in all samples but found a poor correlation between lymphocyte mRNA and total body enzyme function. However, Koch *et al*. and Siest *et al*. were unable to detect CYP3A4 in lymphocytes and used up to 40 ng of total RNA per reaction [178, 181]. Several of these studies measured CYP3A4 in lymphocytes after induction. Weak correlation, if any was found, possibly due to the mechanism of induction. Rifampin activates pregnane X receptor (PXR), which binds to the promoter of CYP3A4. However, PXR expression is tissue-specific and also exhibits interindividual variability [175]. This could explain the negative findings after induction. None of these studies investigated the impact of CYP3A4 genotypes and its expression in lymphocytes with CYP3A4 liver function.

We were able to measure the relative quantities of CYP3A4 in all lymphocyte cell lines. The C_t values of CYP3A4 indicate very low levels present in lymphocytes. *CYP3A4*1/*1G* heterozygotes had not only the most CYP3A4 expression, but also the most CYP3A5 expression, which all had the same genotype. Therefore, CYP3A4 content between cell lines, although significant, but doesn't appear to be meaningful. One would expect to see a gene-dose relationship between cell

line with the heterozygote cells expressing an intermediate level of mRNA, making it impossible for the heterozygotes to express the most CYP3A4. Therefore, there is some other unknown mechanism, other than the *CYP3A4*1G* genotype, causing the *CYP3A4*1/*1G* cells to express the most CYP3A4 and CYP3A5. Perhaps, there are unknown SNPs within the regulation pathway of CYP3A enzymes that is causing the upregulation of both CYP3A4 and CYP3A5, for instance, PXR.

We are confident that all C_ts measured are real and not due to primer/probe degradation. Figure 2.3 shows the qPCR curve shift to the right when less total RNA was added. Also, the ratio C_ts of the 200 ng total RNA to 25 ng total RNA is similar across all genes. Again, indicating that measured C_ts are of templates and changes in C_ts are due to changes in starting template.

In contrast to the qPCR data, protein quantification by immunoblot actually shows *CYP3A4*1G/*1G* has the highest protein content, followed by *CYP3A4*1/*1G*, and not detectable in *CYP3A4*1/*1*. This data is more in line with the gene-dose theory with the heterozygotes expressing an intermediate amount of protein. Interesting, the wild-type lymphocytes exhibit a larger molecular weight band that is present at lower levels in the heterozygote and not present in the *1G homozygote. This led us to perform another immunoblot with a more relevant tissue, human liver microsomes (HLM), to see if this high molecular weight band is also present. This band was not seen in any genotype of HLM samples and

appears to be specific to the lymphocytes. It is unclear at this time what this band is or its clinical relevance, if any. However, it is clear that it is not due to the gene status of *CYP3A4*.

We were unable to measure the activity of CYP3A4 within lymphocytes using a highly sensitive, luminescent method. Cells are incubated with luciferin IPA, which creates luminescence after it is metabolized by CYP3A4, upon addition of the detection reagent. This is a sensitive method to detect minimal activity within the lymphocytes. However, regardless of length of incubation or the concentration of substrate, negligible, if any, activity was measured.

Our study shows that lymphocytes do not appear to be a good source to measure CYP3A4 activity *in vitro*. Our goal was to use lymphocytes as a surrogate human tissue to evaluate the effects of the *CYP3A4*1G SNP*, however, measurements of CYP3A4 mRNA and protein content and CYP3A4 activity in lymphocytes suggest that they are not a reliable surrogate for liver enzyme content and function. Our data confirm that lymphocytes are not able to be used to identify changes in enzymes due to CYP3A genetic variation.

Table 2.1. TaqMan® Probe Information

Protein	Amplicon Length	Location (exons)
CYP3A4 (Hs00604506_m1)	119	2-3
CYP3A5 (Hs01070905_m1)	101	2-3
β-actin (Hs99999903_m1)	171	1
GAPDH (Hs03929097_g1)	58	8

Primer/probes pairs where ordered from Applied Biosystems®. TaqMan® Gene Expression Assay was used with a reporter dye, FAM™ on the 5' end and a nonfluorescent quencher (TAMRA™) located on the 3' end.

Table 2.2. C_ts by Genotype

	CYP3A4	CYP3A5	β-Actin	GAPDH
CYP3A4*1/*1	38.95 ± 0.616	34.02 ± 0.189	14.81 ± 0.147	15.84 ± 0.167
	37.68 ± 0.259	33.85 ± 0.250	13.54 ± 0.015	14.76 ± 0.059
CYP3A4*1/*1G	38.33 ± 0.288	33.06 ± 0.126	16.68 ± 0.224	16.51 ± 0.213
	36.64 ± 0.387	30.61 ± 0.214	13.84 ± 0.282	14.86 ± 0.577
CYP3A4*1G/*1G	38.85 ± 0.315	34.80 ± 0.355	16.07 ± 0.125	16.32 ± 0.200
	37.29 ± 0.367	34.27 ± 0.161	14.05 ± 0.139	14.78 ± 0.154

Two qPCR runs were completed per cell line (each row represents one run). C_t is defined as ten standard deviations above the average background level.

Table 2.3. C_ts of Template Dilution

	CYP3A4	CYP3A5	GAPDH	β-actin
200 ng	38.91 ± 0.54	34.32 ± 0.28	15.79 ± 0.11	20.36 ± 0.11
25 ng	44.68 ± 1.60	39.46 ± 0.84	20.05 ± 0.21	24.89 ± 0.09
Ratio 200 ng/25 ng	0.871	0.870	0.788	0.818

Each qPCR run in quadruplicate. C_t is defined as ten standard deviations above the average background level.

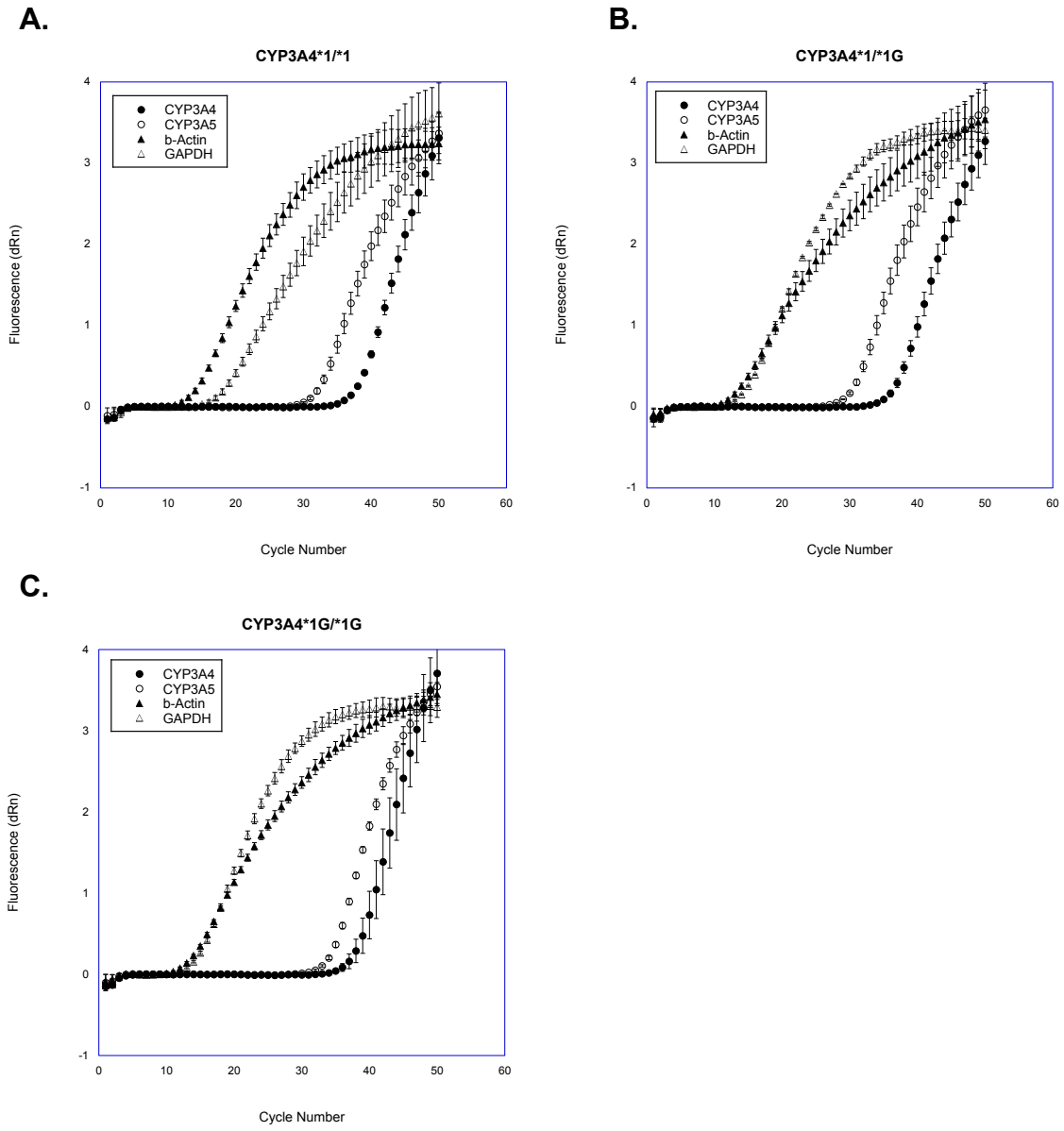


Figure 2.1. qPCR Traces of Lymphocytes. **A.** CYP3A4*1/*1 lymphocytes **B.** CYP3A4*1/*1G lymphocytes **C.** CYP3A4*1G/*1G lymphocytes. Traces (n=4) are representative. Solid circles are CYP3A4 amplicons, empty circles are CYP3A5 amplicons, solid triangles are β-actin amplicons, and empty triangles are GAPDH amplicons. Fluorescence (dRN) is the reporter signal normalized to a passive reference dye, ROX™.

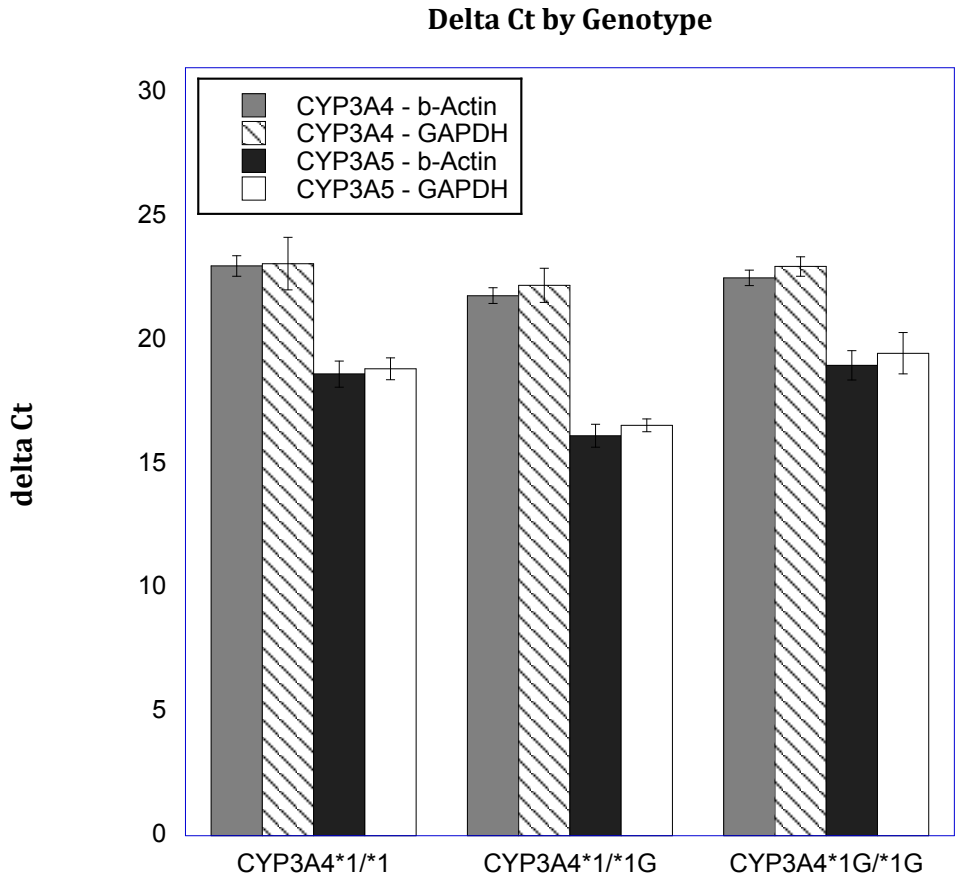


Figure 2.2. CYP3A4 and CYP3A4 Normalized Expression Levels. Each gene is normalized to housekeeping genes, as denoted in the figure legend. Lower values correlate to more mRNA expression of the gene of interest (CYP3A4 or CYP3A5).

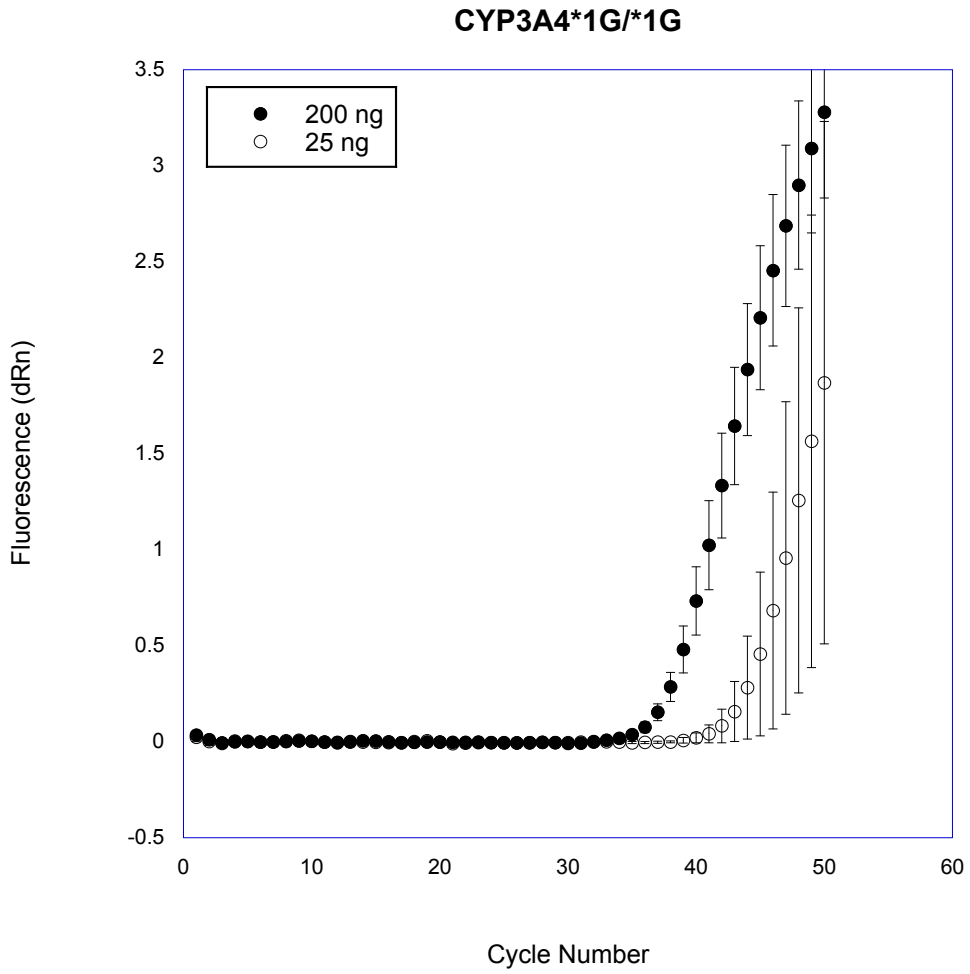
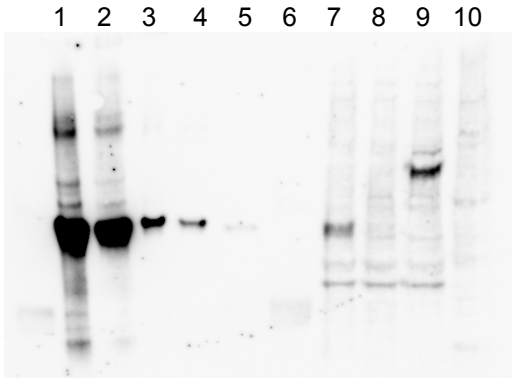
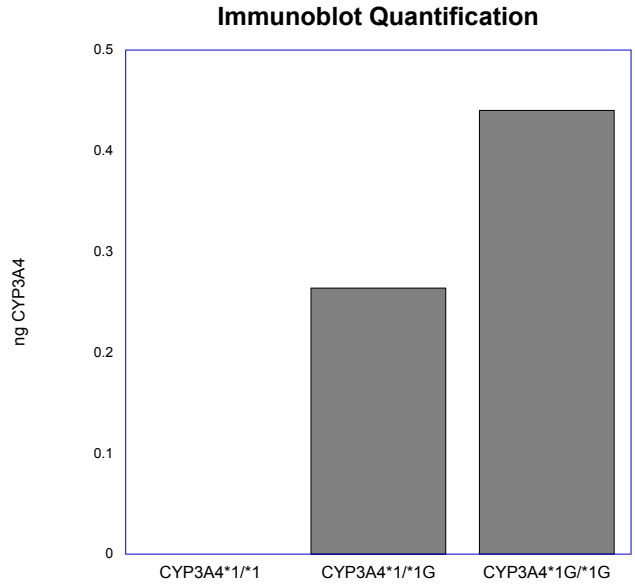


Figure 2.3. qPCR Traces of Template Dilution. qPCR traces of 200 ng (solid circles) and 25 ng (empty circles) of total RNA added per reaction. Fluorescence (dRN) is the reporter signal normalized to a passive reference dye, ROX™.

A.



B.



C.

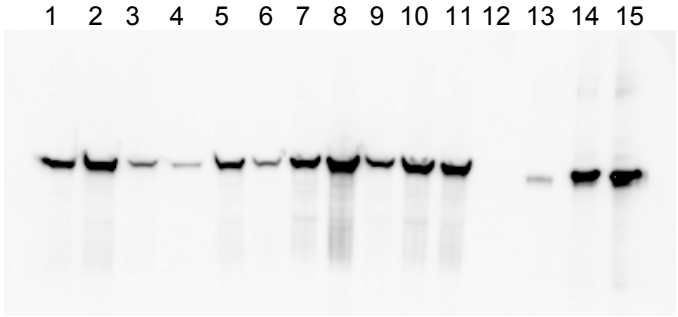


Figure 2.4. Immunoblot and Quantification CYP3A4 Protein. **A.** Lymphocyte CYP3A4 immunoblot. Standards lane 1 (10 ng), 2 (5 ng), 3(1 ng), 4 (0.5 ng), 5 (0.25 ng). Lymphocytes lanes 7 (CYP3A*1G/*1G), 8 (CYP3A4*1/*1G), and 9 (CYP3A4*1/*1). Negative control, Sf9 insect cells, lane 10.. **B.** CYP3A4 protein quantitation stratified by genotype of lymphocyte cell lines. **C**. HLM CYP3A4 immunoblot. Lanes 1 and 2 are CYP3A4*1G/*1G samples, lanes 3-7 are CYP3A4*1/*1G samples and lanes 8-11 are CYP3A4*1/*1 samples. Standards are in Lanes 13(1 ng), 14 (5 ng), and 15 (10 ng).

Chapter 3. Effect of the Genetic Variant *CYP3A4*1G* in Human Liver Microsomes

3.A. Introduction

CYP3A4 is drug-metabolizing enzyme highly expressed in the liver and intestine [97]. CYP3A4 metabolizes about 50% of all medications available on the market, comprised of more than 120 different drugs from therapeutic classes that include antihistamines, immunosuppressive agents, benzodiazepines, and HIV protease inhibitors [99, 131]. Up to 40-fold variability has been reported in interindividual CYP3A4 activity [112, 155, 156] and up to 90% is predicted to be due to genetic factors [154]. Because of the huge diversity of CYP3A4 substrates, genetic variability can have a large clinical impact in drug response and toxicity [30]. Pharmacogenomic studies aim to identify these genetic variations and determine their resulting changes in activity.

Probe drug studies are the gold standard in *in vivo* phenotyping of pharmacogenetic variation as they provide direct predictions of alterations in pharmacokinetic parameters, however, these studies have considerable disadvantages (discussed earlier in Chapter 1 section A.ii.). Therefore, investigators also try to evaluate pharmacogenetic variation using *in vitro* methods [187, 188]. One of these widely accepted methods is using human liver microsomes (HLM) [189]. HLMs are generated from liver samples, obtained by liver biopsies or organ donation. Liver samples undergo several cycles of homogenation and centrifugation to form vesicles from isolated endoplasmic reticulum. Because CYPs are microsomal proteins, HLMs contain concentrated levels of CYPs. Liver microsomes enable one to make pharmacokinetic

estimations (rate of metabolism, intrinsic clearance) that are scalable to whole liver and whole body predictions, without the need to administer medications *in vivo* and gather blood and urine samples. For instance, HLMs were first identified to give good predictions of intrinsic clearance in rats [41, 190-192]. Correlations were soon made between clearances measured in HLMs and those measured in an *in vivo* pharmacokinetic study [187, 193, 194]. In order to best estimate intrinsic clearance, it is important to maintain incubations conditions similar to those seen *in vivo*. Specifically, incubation times need to be such that product formation is linear (initial rate is constant) over the duration and drug concentrations are comparable to clinical concentrations [187]. Scaling from HLMs to whole body deviate the most when metabolism occurs in organs other than the liver, for instance, the intestines, or when metabolism occurs by routes other than CYP oxidation [187, 195]. This scenario would lead to an underestimate of total clearance [187]. HLMs are commonly used in research as well as drug development [196]. They are inexpensive, convenient, readily available, and create reproducible data [197]. When stored at -80°C, microsomes retain their activity for years [198].

There are disadvantages to using microsomes as an alternative for *in vivo* data. Disease and medication histories of the subjects from whom the livers are obtained are often unknown or incomplete. Disease state, such as diabetes mellitus, can cause decreased CYP3A4 expression and activity [199]. Length of time before organ procurement can also affect CYP expression; increased

cytokine release, due to inflammation and infection, as well as decreased hormone secretion leads to down regulation of CYPs [30, 154, 200]. Concurrent medications can cause inhibition or induction and need to be incorporated into data analysis. Also, organ collection protocols, tissue storage conditions, and microsomal preparations all can have an effect on microsomal enzyme activity. Because of this, it has reported that *in vitro* interindividual variability is larger than *in vivo* interindividual variability [129].

Our study aimed to investigate the any change in activity due to the *CYP3A4*1G* genetic variant *in vitro* using HLMs that have been previously genotyped. CYP3A4 activity in HLM was measured using a selective CYP3A4 substrate with minimal/no substrate overlap with CYP3A5. Our goal was to make genotype-phenotype associations in the HLMs to assess *CYP3A4*1G* activity.

3.B. Materials and Methods

3.B.i. Human Liver Microsomes

Human liver microsomes (n=324) were obtained from University of Washington, School of Pharmacy Human Liver Tissue Bank (Seattle, WA). The University of Washington made all human liver microsomes. Liver tissue was homogenized using a Bead Ruptor. Homogenate was centrifuged at 15,000g for 30 min. Centrifuge supernatant at 120,000g for 70 min. The pellet was resuspended in wash buffer and homogenized with a glass homogenizer and centrifuged at 120,000 g for 70 min. Pellet resuspended in wash buffer with a glass

homogenizer and stored at -80°C. Livers were genotyped in Debbie Nickerson's Laboratory in the Department of Genome Sciences University of Washington (Seattle, WA). Genotyping was determined via PGRNseq Platform (University of Washington). PGRNseq is a next-generation sequencing platform that sequences coding regions, adjacent noncoding regions, 2kb upstream, and 1kb downstream in 84 genes Very Important Pharmacogenes.

3.B.ii. CYP3A4 Activity in Human Liver Microsomes

CYP3A4 activity in HLMs (n=64) was determined with the P450-Glo™ kit (Promega) according to manufacturer's specifications. Five μg of total protein were preincubated in microfuge tubes with 4x P450-Glo™ substrate (luciferin IPA) and KPO_4 buffer for 10 min in a water bath at 37°C. An equal volume of 2mM NADPH was added to initiate the reaction and HLMs were, again, incubated for 10 min in a water bath at 37°C. An equal volume of Luciferin Detection Reagent was added and tubes were placed on an orbital shaker for 15-20 min at room temp. All incubations were completed in triplicate. Pooled HLMs were used as a positive control in every incubation set (in duplicate). Solution was transferred to a white, untreated 96-well plate and luminescence detected on a SynergyMX microplate reader (Biotek, Winooski, VT) with an integration time of 1 second/well. Optimization of HLM incubation conditions was completed using pooled HLM (Xenotech, Lenexa, KS).

3.B.iii. CYP3A4 Protein Quantitation in Human Liver Microsomes

Protein quantitation was performed by immunoblot. Total microsomal protein (10 μg) was resolved on a Tris-Hepes NH 4-20% gel (NuSep). Protein was transferred to nitrocellulose membranes and blocked with 5% milk for 1 hour. The membrane incubated with primary anti-CYP3A4 antibody (Abnova) (1:1,000 dilution) on a rocker overnight at 4°C. Secondary anti-mouse antibody was added (1:25,000 dilution) for 1 hour at room temp. Membrane was developed using the West Femto Kit (Thermo Scientific™) and imaged on an LAS-3000 camera (Fujifilm).

3.B.iv. Data Analysis

An ANOVA was performed to test the differences between the HLM genotypes in both activity and protein quantitation, α=0.05 (KaleidaGraph). A multivariable regression was completed to test the significance of each variable on CYP3A4 activity. Variables tested were: protein content, genotype, age, gender, race, taking a 3A4 inducer, taking a 3A4 inhibitor, liver pathology, cause of death, height, weight, ICU time, liver ischemia, organ trauma, smoking status liver lab results, and pathology. Missing data was not included in the analysis (StatsPlus, Alexandria, VA)

3.C. Results

3.C.i Optimization of Microsomal Incubation Conditions

HLMs are precious samples, some with extremely limited protein, so we optimized the HLM assay using commercially available pooled HLMs (Figure 3.1). First, microsomal protein per incubation was titrated to determine the linear range of product formation (Figure 3.1A and 3.1B); we found that product formation was linear between 1.25 and 10 μg total protein per reaction. The substrate, luciferin IPA, is provided from Promega in 100% DMSO, however, DMSO is a known inhibitor of CYPs [201]. Therefore, we next optimized DMSO concentration over a range of substrate concentrations (Figure 3.1C) and found the largest response using a substrate concentration of 8 μM in 0.25% DMSO. Finally, we optimized for time to assure linear product formation (Figure 3.1D) and found that product formation is linear between 5 and 20 min. We concluded from the optimization experiments that incubations with liver bank HLMs be carried out with 5 μg total protein and 8 μM final substrate concentration for 10 min.

3.C.ii. Subject Demographics

Demographics are listed in Table 3.1. Males made up 56.8% and Caucasian was the most common ethnicity at 95.4%. Other ethnicities are reported at less than 3%. At the time of writing this thesis, the only data received on genotypes was for 64 livers; 48 are wild-type (75%), 14 are heterozygote (21.9%), and 2 are homozygote for the *1G SNP (3.1%). Medications were not reported for all

samples, however, 12 were reported to be taking a CYP3A4 inducer (3.7%) and 18 were reported to be taking a CYP3A4 inhibitor (5.6%).

3.C.iii. Analysis of CYP3A4 Activity

Total CYP3A4 metabolic rate ranged from non-detectable to 10.3 pmol/μg protein/min (Figure 3.2) in 324 livers, demonstrating a huge variability in the CYP3A4 activity. Pooled HLMs were used as a positive control. The average rate is 1.64 ± 0.30 pmol/μg protein/min, making the interday coefficient of variation 18%.

The CYP3A4 metabolic rates of 64 genotyped livers are stratified by genotype: *CYP3A4*1/*1* (n=48), *CYP3A4*1/*1G* (n=14), and *CYP3A4*1G/*1G* (n=2) (Figure 3.3). There was no statistical difference between any genotypes (p=0.519), however, there was a trend towards increased activity in *CYP3A4*1G* homozygous individuals, although numbers were small.

A linear regression analysis was performed with all data/variables reported from the liver bank with the HLMs. All variables, including genotype and gender, did not significantly impact the CYP3A4 rate.

3.4. Discussion

The demographics of the liver samples are not very diverse. The majority of the samples are Caucasian (95.4%), male (56.8%), and procured from patients who

were between the ages of 40 and 59 (32.4%) (all are independent variables). There are few samples that are classified as Black (n=5), Asian (n=1), and Hispanic (n=2). Therefore, it is difficult to tease out an effect from ethnicity due to limited sample number in this liver bank. Patients on medications that induce or inhibit CYP3A4 were also few (n=7 and 5, respectively), again, making it difficult to find significance on its effect on CYP3A4 metabolism. Patients classified as taking medications that induce CYP3A4 were taking, phenytoin, a strong inducer, and patients taking CYP3A4 inhibitors were taking amiodarone and cyclosporine, both weak inhibitors, and erythromycin, a moderate inhibitor. No donors were taking a strong inhibitor. There were several samples that had no report of medications, reporting of medications could have accounted for some interindividual variability. Regardless, all variables reported had no significant effect on the measured CYP3A4 metabolism rate in human liver microsomes. This was a bit unexpected as it is reported that females can express up to 2-fold higher levels of CYP3A4 *in vivo* [136]. Also, there appears to be a trend that liver microsomes with higher protein contents tend to have a higher CYP3A4 metabolism rate, even after normalized to protein content (Figure 3.5). Although, again, this is statistically insignificant when analyzed in a multivariable regression.

We next further examined the effect of only the *CYP3A4*1G* genotype on CYP3A4 metabolism. The average rate was determined of each genotype and an ANOVA was performed to identify if there was any difference between the three average rates. This, too, was not statistically significant (p=0.51898).

However, there is a trend toward increasing function of the *CYP3A4*1G* genotype. With only 64 genotyped HLMs, only two were identified as *CYP3A4*1G/*1G*. When the rest of the liver bank is genotyped and included in the statistical analysis, there should be more homozygote *1G samples identified, resulting in an increase in statistical power. This may help determine if there is truly an increase in enzyme activity due to *CYP3A4*1G* genotype.

Our current data do not allow us to make a definite prediction of *CYP3A4*1G* impact on CYP3A4 metabolism. There was no statistical significance between *CYP3A4*1G* genotype and CYP3A4 expression or activity. However, at the writing of this thesis, of the 324 microsomes with activity data, we only have genotypes reported for 64 microsomes. When the genotypes of the other 260 genotypes are incorporated in the analysis, perhaps significance can be teased out with the higher sample numbers and higher power. Until then, our data suggest that *CYP3A4*1G* has little significance on total metabolism and does not account for measured interindividual variability.

Table 3.1. Human Liver Microsome Demographics

	Percentage	n
Gender		
Male	56.8%	184
Female	42.0%	136
Unreported	1.2%	4
Ethnicity		
Caucasian	95.4%	309
Black	2.8%	9
Hispanic	0.6%	2
Asian	0.3%	1
Unknown	0.9%	3
Age		
0 – 19	22.2%	72
20 – 39	17.3%	56
40 – 59	32.4%	105
60 – 79	20.1%	65
80 – 99	1.5%	5
Unknown	6.5%	21
Genotype		
CYP3A4*1/*1	14.5%	47
CYP3A4*1/*1G	4.3%	14
CYP3A4*1G/*1G	0.6%	2
Unknown	80.6	261
CYP3A4 Inducers		
No	39.8%	129
Yes	3.7%	12
Unknown	56.5%	183
CYP3A4 Inhibitors		
No	38.3%	124
Yes	5.6%	18
Unknown	56.2%	182

Human liver microsomes demographics reported from human liver bank (University of Washington) (n = 324).

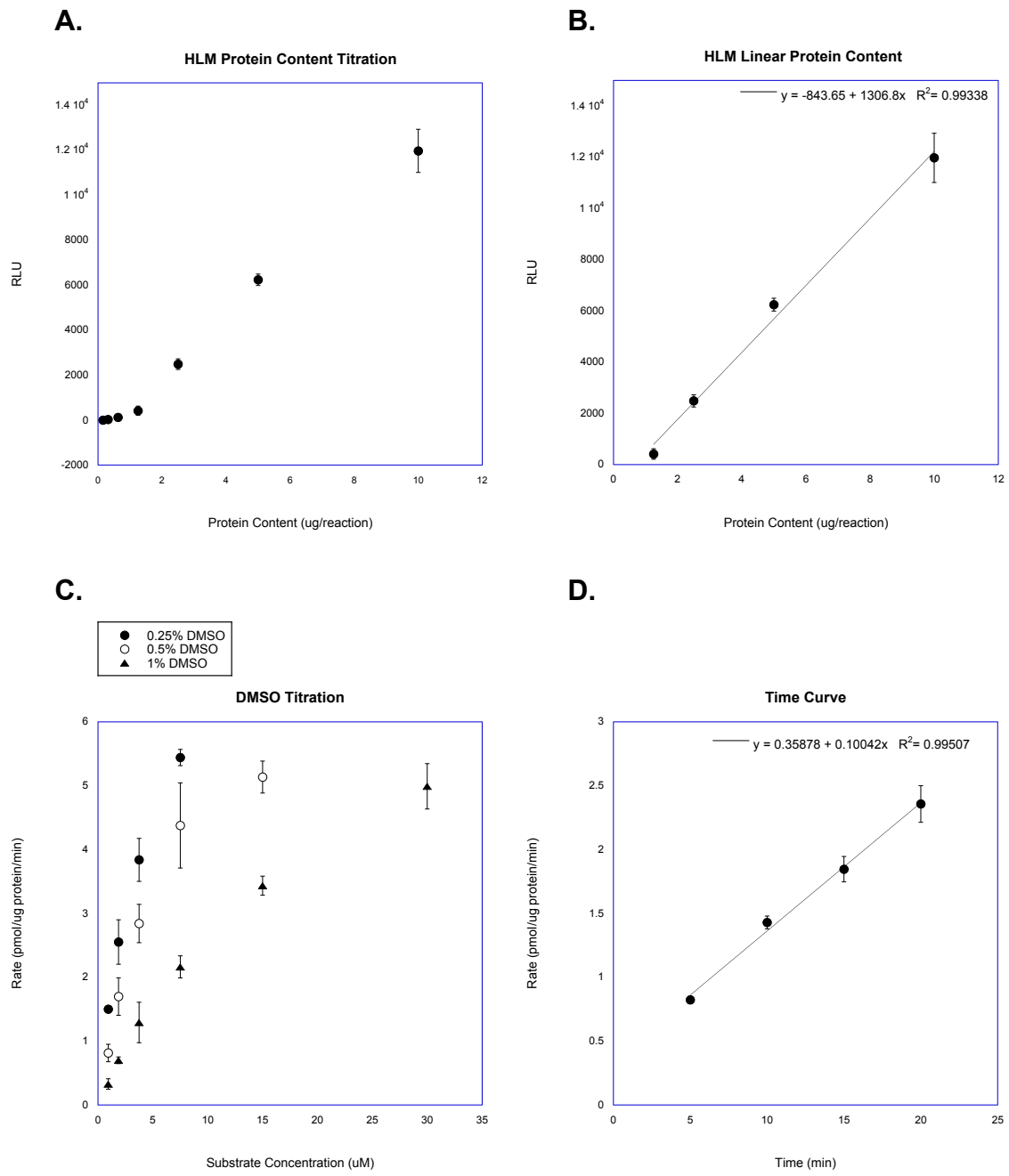


Figure 3.1. Optimization of CYP3A4 Activity in Pooled Human Liver Microsomes. **A.** Titration curve of total protein content of pooled. **B.** Linear range of total protein content titration curve. **C.** DMSO titration. **D.** Time curve completed to determine linear range of product formation.

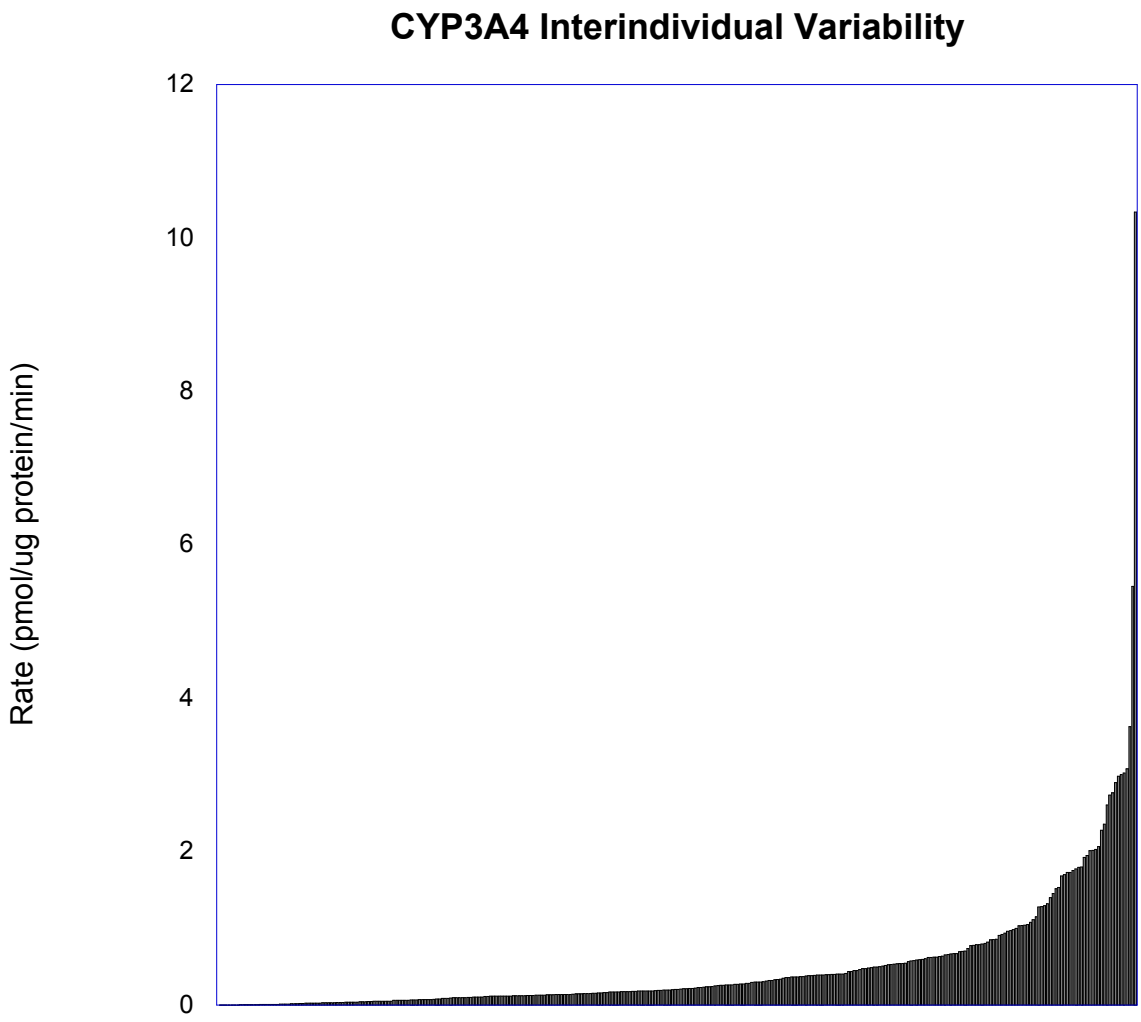


Figure 3.2. **CYP3A4 Interindividual Variability Measured in Human Liver Microsomes.** 5 μg of liver microsome total protein was incubated with 8 μM luciferin IPA for 10 minutes. Detection reagent was added and luminescence was quantitated on a microplate reader (n=324).

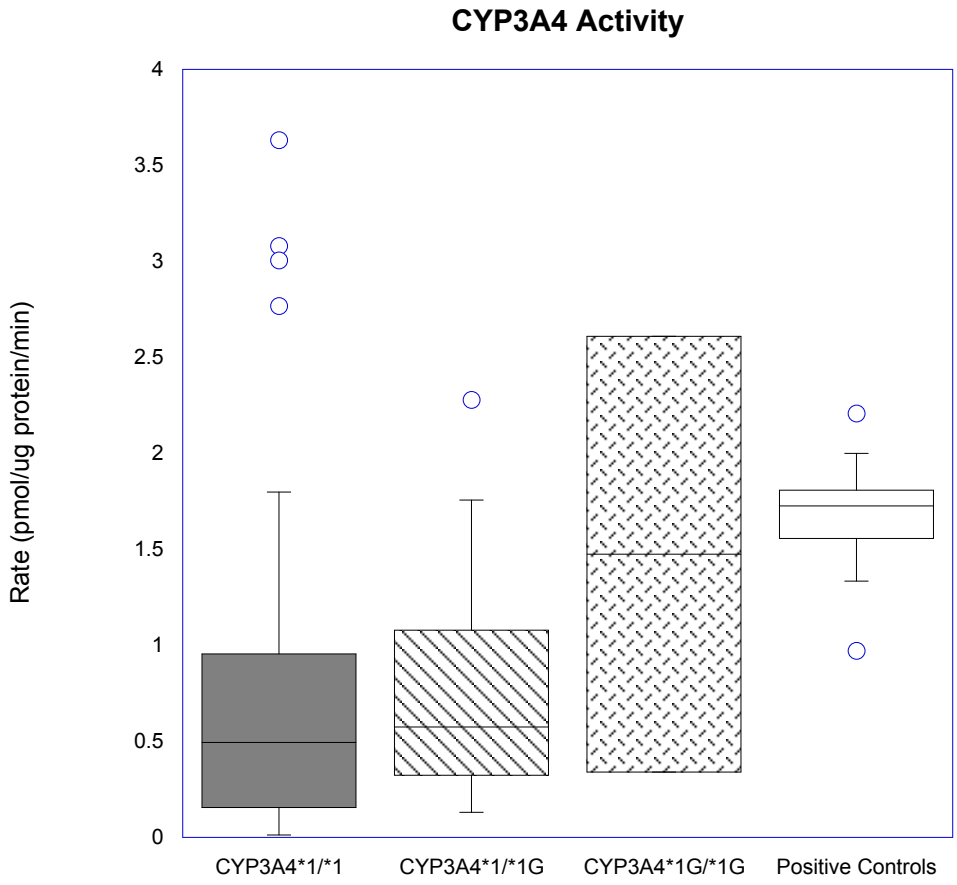
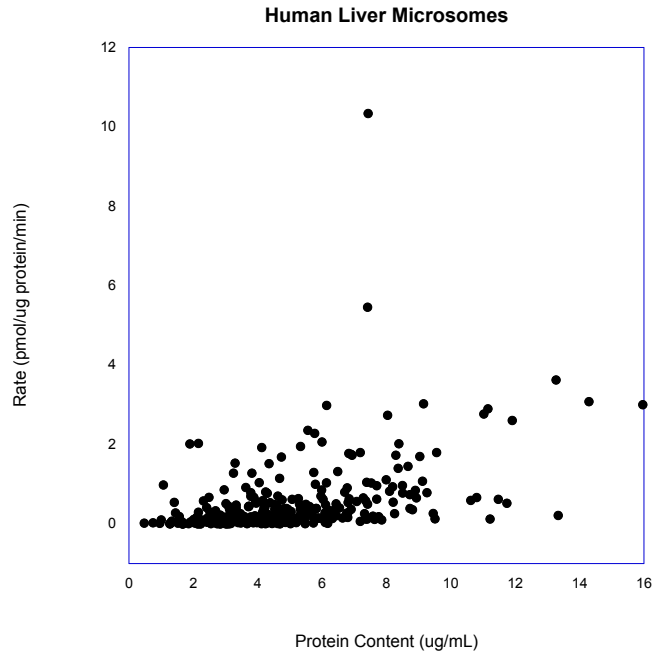


Figure 3.3. CYP3A4 Activity in Human Liver Microsomes. HLMs were stratified by CYP3A4 genotype (CYP3A4*1/*1 n=48; CYP3A4*1/*1G n=14; CYP3A4*1G/*1G n=2). Positive controls were run in duplicate. An ANOVA found no statistical differences between genotype (p=0.51898).

A.



B.

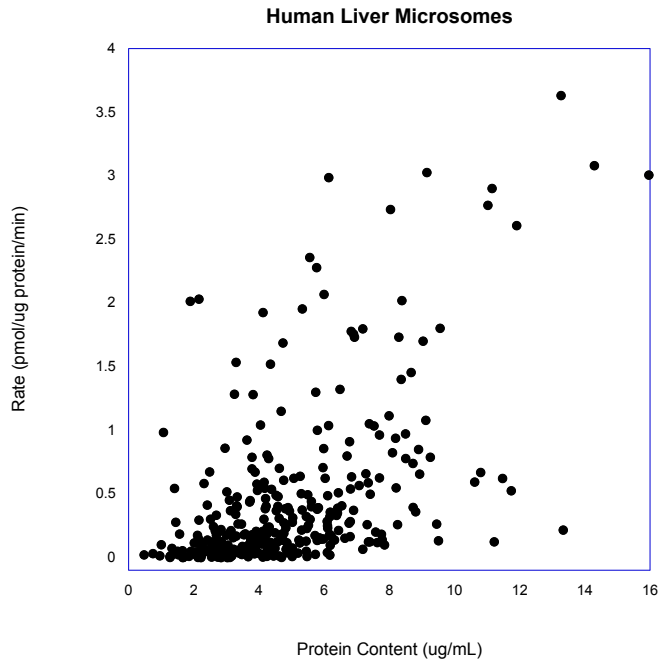


Figure 3.5. Relationship Between Human Liver Microsome Total Protein Content and CYP3A4 Rate. A. Although there is no significant statistical relationship between total protein content and CYP3A4 metabolism rate, there is a trend between higher protein content and increased CYP3A4 rate. (n=324) **B.** Data points with rates greater than 5 pmol/ug protein/min were removed (n=322) (R=0.43).

Chapter IV: Summary and Future Directions

The goal of my research project was to decipher the functional consequence of the genetic variant *CYP3A4*1G*. I used *in vitro* models to assess CYP3A4 mRNA and protein expression and activity in lymphocyte cells and human liver microsomes.

We hoped to use lymphocyte cell lines, with the genotypes *CYP3A4*1/*1, CYP3A4*1/*1G,* and *CYP3A4*1G/*1G*, as surrogates of CYP3A4 drug metabolism. This convenient, limitless resource with known genotypes would have been ideal to study the SNP of interest. Also, the ability to quantitate CYP3A4 mRNA, protein, and activity could have huge implications in clinical field with the ability to phenotype patients with a single blood draw. Despite using a sensitive, luminescent detection method to measure CYP3A4 activity, we were unable to observe CYP3A4 activity in the lymphocytes. Therefore, lymphocytes do not make good surrogate markers for drug metabolism and cannot be used to measure a patients' CYP3A4 drug metabolism rate and to make any conclusions as the functional consequence of the *CYP3A4*1G* genetic variant.

The second part of my research involved using human liver microsomes to assess the functional consequence of *CYP3A4*1G* variant. CYP3A4 activities were measured in 324 human liver microsomes (HLMs). At the time of writing this thesis, only 64 of these HLMs have been genotyped: 48 were homozygous wild-type *CYP3A4*1/*1*, 14 heterozygous *CYP3A4*1/*1G*, and 2 homozygous for *CYP3A4*1G/*1G*. Although a trend was observed towards increased activity in

the *CYP3A4*1G* containing livers, a multivariable regression and ANOVA both found no significance of the *CYP3A4*1G* genetic variant. Until the genotypes of the remaining HLMs are known, there is not enough power to determine the effect of *CYP3A4*1G.*

In addition to incorporating the genotype of the remaining livers into the analysis, we will also normalize to the CYP3A4 protein levels, quantified by mass spectrometry, both completed at the University of Washington, as soon as the data collection is completed. However, in future work, I would measure the protein content of known CYP3A4 transcription factors, pregnane X receptor, constitutive androstane receptor, retinoid receptor, and hepatocyte nuclear factor in the human liver microsome samples [202, 203]. This would allow us to account for any of the *CYP3A4* interindividual variability due to increased or decreased expression of transcription factors and may help us better ascertain any changes in function due to genetic variations in CYP3A4.

Other studies have found that *CYP3A4*1G* either causes increased [137, 138, 140] or decreased [116, 118-120] clearance *in vivo*. These mixed results could arise from the contribution of other CYP3A proteins. CYP3A7 may have minor impact as it is only expressed in 10% of adults, however, it can have large impact in CYP3A drug metabolism in those individuals as CYP3A7 has been reported to contribute up to 40% of CYP3A content [145-147]. *CYP3A5* genotype could play a larger role in interindividual variability in CYP3A drug metabolism. In those who

express CYP3A5, CYP3A5 makes up 50% of CYP3A content [100]. Because of the linkage disequilibrium found between *CYP3A4*1G* and *CYP3A5*1* found in HapMap populations, it is difficult to identify the functional consequence of *CYP3A4*1G*. However, a novel break in the linkage of *CYP3A4*1G* and *CYP3A5*3* has been uncovered in the Confederated Salish and Kootenai Tribes. Due to the high *CYP3A5*3* allele frequency in this population, many would carry both the *CYP3A4*1G* allele and the *CYP3A5*3* allele. Studying this population, would allow us to assay for changes in CYP3A4 drug metabolism without the contribution from CYP3A5. Therefore, our laboratory has a proposed study to evaluate the *CYP3A4*1G* genetic variant in the CSKT population using an oral, subtherapeutic dose of midazolam, a CYP3A4 probe drug. Blood and urine will be collected to measure midazolam and its metabolite over time. This study will shed light on the *CYP3A4*1G* functional consequence unlike any other study has been able to do; we will be able to unequivocally assay for CYP3A4*1G metabolism.

In conclusion, changes in the CYP3A4 metabolism rate due to the *1G SNP can have wide implications, especially in Japanese, Chinese, and CSKT populations, where the *1G allele has been reported in high frequencies [90]. Because CYP3A4 is responsible for metabolizing up to 50% of medications currently on the market [129, 142-144], any changes in its metabolism rate will affect the clearance of several medications. Also, any changes in CYP3A4 metabolism rates can be exaggerated when inherited with nonfunctional CYP3A5, like that

seen in the CSKT population. It is important to identify the effect of the *CYP3A4*1G* genetic variant to better predict pharmacokinetic and pharmacodynamic changes to lead to increased drug efficacy and decreased toxicity.

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